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# Clonogenicity of Human Promyelocytic Leukemia Cells Enlarged and Multinucleated by Treatment with Cytochalasin B and their Potential as Targets for Improved Chemical and Physical Therapeutic Approaches

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## **Introduction**

### *What is Cancer?*

Our bodies are able to renew and repair themselves, but when the mechanisms that control these processes are defective or not working, cancer can develop. Cancer, in simplest terms, is a disease of tissue renewal. Tissue involution and tissue expansion are controlled by various cellular processes and when the balance of these processes is disturbed, disease results.

Degeneration/involution results when cell losses exceed renewal. Tissue expansion, hyperplasia, or neoplasia result when cell renewal exceeds cell losses (Pelengaris et al., 2006). Cancer cells are defined by two properties: genetic alterations allow them to divide and survive when they should not, and they invade and colonize areas reserved for other cells.

Cancer cells clustered together in a mass are called tumors and two types of tumors result from tissue expansion. A benign tumor proliferates, does not invade surrounding tissues, and can usually be removed cleanly. A malignant tumor proliferates, invades surrounding tissue, and spreads to distant sites. Malignant tumors break from the primary tumor, enter the bloodstream or lymphatic vessels, and form secondary tumors called metastases (Alberts et al., 2004). Genetic instability of cancer cells leads to an accumulation of mutations. These accumulating mutations cause the developing cancer cells to progress toward increasing abnormality. These mutations usually occur over a period of many years, which is why cancer is typically a disease of old age.

People fear being diagnosed with cancer. Early diagnosis and removal of the tumor before it has invaded provide the best hope for cure. Once cancer begins to spread, chemotherapy and radiation provide palliative, and sometimes curative, therapy. Initially, therapies can provide positive effects, but the cancer can recur when a tumor becomes drug-resistant. We have to be able to understand regulating cell growth in order to be able to control tumor growth and improve our ability to treat these diseases.

### *Cancer Cells*

Cancer cells have several key abnormalities. They have a reduced dependence on signals from other cells, usually due to mutations in the cell signaling pathways. Cancer cells are unlikely to kill themselves by apoptosis because of mutations in the genes that regulate the intracellular death program. When the DNA of a normal cell is damaged, a checkpoint mechanism causes it to stop dividing until the damaged DNA is repaired. If the DNA damage is not repaired the cell is programmed to die by apoptosis. This, and other checkpoint mechanisms, are defective in cancer cells.

Furthermore, cancer cells proliferate indefinitely. Normal cells divide a limited number of times because the telomeres on the ends of their chromosomes become too short. Cancer cells, however, reactivate the production of the telomerase enzyme, which maintains telomere length, so they can divide indefinitely. Because they are genetically unstable, cancer cells have a greatly increased mutation rate. They are also abnormally invasive because they lack

cadherins, or other specific cell-adhesion molecules, that hold normal cells in their place. Finally, cancer cells form metastases by surviving and proliferating in foreign tissues, whereas most normal cells die when they are misplaced (Alberts et al., 2004).

We think that cancer cells may be showing loss of actin microfilament check-point control of entry into the cell cycle similar to what is seen with yeast mutants. Cancer cells may lack morphological and geometrical controls that prevent normal cells from entering the cell cycle if their size, shape, and attachments to basement membrane or other supporting growth surfaces are not correct. Indeed, one characteristic morphological phenotype of cancer cells is their ability to grow in suspension medium or in semi-solid medium under conditions which normal cells will not grow. Another characteristic feature of cancer cells in culture is their loss of density-dependent inhibition of cell division, a property called contact-inhibition of cell division. Cancer cells will continue to divide even when in a confluent layer and will pile up on one another making a transformed colony of cells. Normal cells do not exhibit this behavior. Normal cells will divide when seeded in dilute concentration on a surface, but will stop dividing when they contact each other.

Normal mammalian cells, like yeast cells, show behavior that is mediated by cell morphology presumably linked to the cell cytoskeleton. Mammalian cancer cells may, like mutant yeast cells, exhibit defects in these morphological controls of cell behavior. Two key morphological features of cancer cells are (1) they exhibit loss of density-dependent inhibition of cell division when they reach

confluency in monolayer culture. They continue to divide, even though they are no longer in contact with the growth substratum, forming foci of over-lapping cancer cell nodules. (2) They exhibit loss of contact inhibition of cell migration allowing them not only to continue to divide when they contact one another, but also to continue to migrate even though they are in confluent contact with one another. These are characteristic morphological features of the transformed phenotype. In the section that follows: Potential Actin-Mediated Morphological Check-point Control of Cell Cycle Entry, I will elaborate in detail on the morphological checkpoint control features of cancer cells that we propose are the basis for the effects of microfilament-directed agents on cancer cells that cause them to behave differently than normal cells when their actin cytoskeleton is disrupted.

Over a hundred genes have been identified that can change normal tissue into cancerous tissue. Oncogenes, the dominant transforming genes, encode proteins involved in signal transduction and/or cell cycle regulation. An oncogene is an over-expressed and altered form of the normal cellular gene called a proto-oncogene. It has dominant transforming properties, in that a single copy of the altered sequence can transform a cell, even in the presence of a normal copy of the proto-oncogene (McKinnell et al., 1998). Proto-oncogenes encode proteins that are involved in signaling cascades and, along with oncogenes, are classified according to their functional role, either as growth factors, receptors, tyrosine kinases, apoptosis pathway regulators, GTP-binding proteins, serine/threonine kinases, or nuclear proteins/transcription factors.

Normal cells surrounding initiated cells may suppress the outgrowth of the cell containing the oncogene. When a tumor cell and a normal cell are fused, the hybrid cell usually exhibits a normal phenotype demonstrating the presence of a dominant tumor suppressor gene in the normal cell that is absent or non-functional in the transformed cell. Suppressor genes within a normal cell encode proteins that negatively regulate cell growth and also function in signal transduction and cell cycle regulation. Some tumor suppressors guard the cell against DNA damage and help repair the damage before the cell divides. Tumor suppressors can arrest growth at the first gap phase ( $G_1$ ) and at the second gap phase ( $G_2$ ) of the cell cycle. The integrity of the chromosomes is checked at these points before the cell proceeds into DNA replication (S phase) or mitosis (M phase) (Alberts et al., 2004). When tumor suppressor function is lost, these checkpoints do not function correctly and cells with damaged DNA can survive and can enter the cell cycle to replicate and amplify the genetic anomalies.

Hundreds of different tumor types arise from almost every tissue and every organ in the body, making it very complicated to classify cancers. The fact that cancer cells show progressive changes toward increasing malignancy and can invade and metastasize to distant organs further complicates the issue. Cancer biologists and oncologists have determined a classification system based on the tissue of origin, regardless of organ location. Carcinomas originate from epithelial cells and typically represent over 80% of diagnosed human cancers. Sarcomas originate in the bone, cartilage, fat, muscle or other connective tissue. Lymphomas and leukemias originate from hematopoietic stem cells that generate

differentiated cells important in the blood including cells of the immune system. Our lab focuses on leukemias, which are cancers that originate in blood-forming tissues, like the bone marrow, and cause large numbers of abnormal blood cells to be produced (Pelengaris et al., 2006). Leukemias secondarily involve the spleen, lymph nodes, and liver. Normal hematopoietic elements are displaced from the marrow by leukemia cells. Leukemia cells then spread and the final reserves for producing functional hematopoietic elements are lost.

*Potential Actin-Mediated Morphological Check-Point Control of Cell Cycle Entry*

Daniel Lew has discovered a morphogenesis checkpoint in yeast cells that monitors the actin cytoskeleton (Lew et al., 1998; Lew, 2000). It implies that spatial and geometric physical controls, not just chemical signal controls, regulate normal cell biology. This morphological checkpoint control of cell cycle progression in yeast may relate to observations in neoplastic cells. Checkpoint controls, as I have mentioned, monitor cell cycle events and delay cell cycle progression if the events are not completed (Lew et al., 1998). Lew found that when the actin cytoskeleton is perturbed, a morphogenesis checkpoint delays nuclear division and bud formation. Bud formation requires a polarized actin cytoskeleton and the delay occurs until a bud is formed. The cell cycle pauses in G2 until the actin can polarize and form a bud (Lew, 2000).

This delay in bud formation along with the cell cycle progression delay prevents the formation of multinucleated cells by monitoring the integrity and organization of the actin cytoskeleton (Lew, 1998). Yeast that are mutant for

morphogenetic checkpoint functions, like neoplastic cells, do not recognize the actin perturbation and do not exit the cell cycle like normal yeast cells and normal mammalian cells. Instead, they continue to divide and become multinucleated. This could imply that if this morphological checkpoint control also functions in mammalian cells, then there might be a mutation in this morphogenesis pathway in mammalian neoplastic cells.

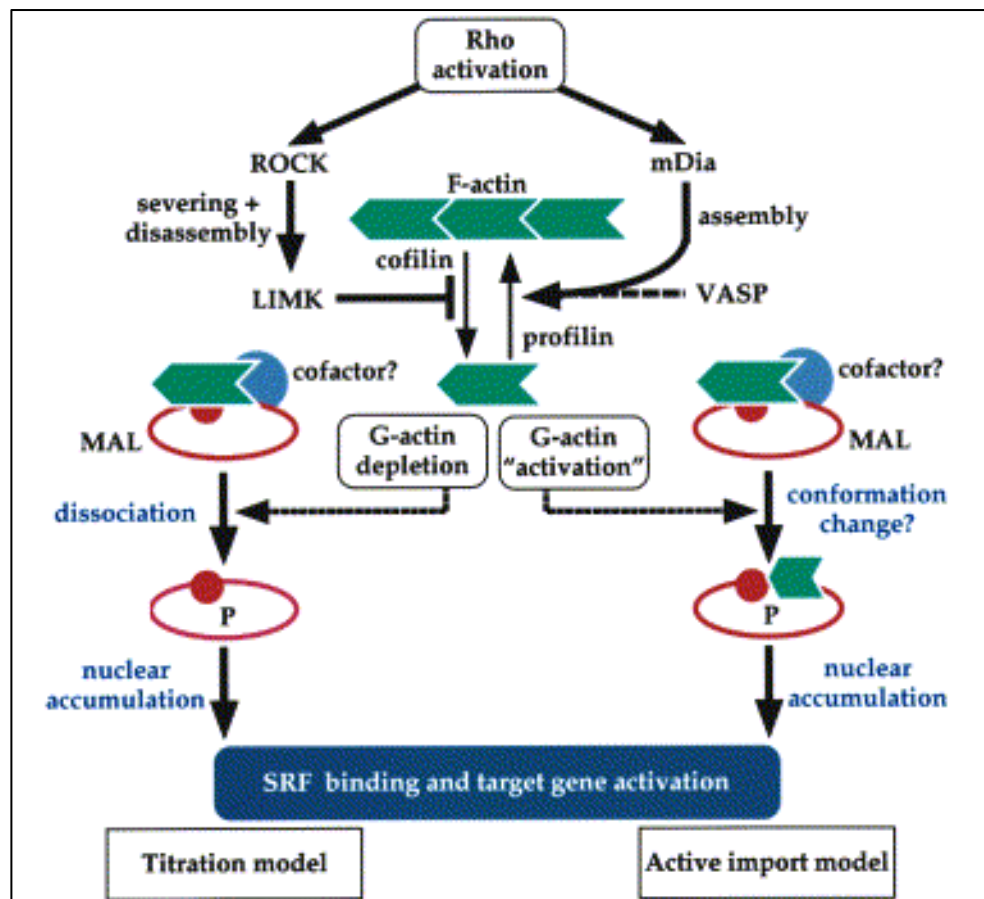
There is evidence to support mechanisms that monitor actin cytoskeleton integrity and organization in cells other than yeast cells. Treisman et al. (2003), have discovered a transcription-modulation pathway whose regulation is controlled by the availability of monomeric actin in mammalian cells.

Unpolymerized actin binds directly to a transcription cofactor in this pathway (Lew et al., 2003). Serum response factor (SRF) is a transcription factor that controls growth-factor-regulated immediate-early (IE) genes, like cytoskeletal actin, and numerous muscle-specific genes. Studies revealed that SRF and actin polymerization have a close connection and that actin is directly involved in the signaling pathway of SRF (Treisman et al., 2002).

Treisman et al. (2003) studied the signaling to SRF via actin. Activated Rho GTPases regulate SRF by their ability to induce actin polymerization by two effector pathways: the ROCK-LIM kinase-cofilin effector pathway that stabilizes F actin (Geneste et al., 2002) and the mDia1 effector pathway that promotes its assembly. ROCK is a Rho-dependent kinase, LIM is an actin binding kinase, and mDia1 is a diaphanous-related formin protein involved in cytokinesis (Treisman et al., 2002). Rho-actin signaling controls the localization of the SRF coactivator



MAL (Treisman et al., 2003). MAL stands for megacaryocytic acute leukemia protein and is a transcription factor. Responding to Rho-induced actin polymerization, MAL is redistributed from the cytoplasm to the nucleus and associates with unpolymerized actin through its conserved amino terminal RPEL (Arginine, Proline, Glutamic Acid, Leucine) motifs (Treisman et al., 2003). It then binds to SRF and induces gene activation. Figure 1 shows the ROCK-LIM and mDia1 effector pathways involving actin.



**Figure 1: The ROCK-LIM and mDia1 effector pathways involving actin.**  
*Source: Treisman, R., Miralles, F., Posern, G., & Zaromytidou, A. (2003). Actin Dynamics Control SRF Activity by Regulation of Its Coactivator MAL. The Journal of Cell Biology, 113, 340.*

An important aspect of this signaling pathway that ties into my research is related to the inactivation of SRF. SRF inactivation reduces the response of immediate-early gene functions associated with this pathway, like actin, and muscle-specific gene expression, which alters cell adhesive properties and causes a non-cell-autonomous gastrulation defect (Treisman et al., 2003). For this reason, Treisman et al. (2003) believe that a specific set of SRF target genes that are involved in cell morphology, adhesion, and movement are controlled by MAL signaling. In my research, when the actin cytoskeleton microfilaments of human neoplastic cells are disrupted with Cytochalasin B and therefore become enlarged and multinucleated, it is possible that SRF is inactivated because of the defect in this pathway and therefore the target genes are not activated properly. This could contribute or cause the properties of multinucleation and lack of cell adhesion seen in the neoplastic cells when their actin cytoskeleton is disrupted with microfilament-directed agents such as cytochalasins or latrunculins. Normal mononucleated cells have no defects in the MAL signaling pathway.

Some of the GTPases involved in, or parallel to, the MAL signaling pathway may be a family of guanine nucleotide binding proteins called septins. Septins are GTPases required for cytokinesis. Loss of septin function results in multinucleation (Kinoshita et al., 2002). Septin's nature is uncertain, but it is believed to be an essential filament beneath the cleavage furrow to ensure cytokinesis completion (Kinoshita et al., 2002). Kinoshita et al. found that septins may be required for normal actin organization. They also found that septins play

a role in other cellular processes including exocytosis, apoptosis, leukemogenesis and carcinogenesis, and neurodegeneration (Kinoshita et al., 2002).

Osaka et al. (1999) found that the gene designated as MLL is frequently rearranged in patients with mixed-lineage leukemia who have been treated with topoisomerase II inhibitors. Using cDNA from the cells of a leukemia patient, they found the cDNA to code for another member of the septin family, which they named megakaryocyte stimulating factor, or MSF. Analyzing MSF further may help to determine the function of MLL partner genes in leukemia (Osaka et al., 1999). Other studies have found that the MLL septin-like fusion gene, MSF, also plays a role in breast and ovarian cancers (Kalikin et al., 2000 and Russell et al., 2000).

### *Cytochalasin B*

Our lab's focus is on exploiting enlarged multinucleated leukemia cells that are produced when leukemia cells are treated with microfilament directed agents, specifically with Cytochalasin B. We propose that enlarged multinucleated cells may be particularly susceptible to therapy, either by damage to their excess nuclei using DNA-directed anti-neoplastic agents or X-Ray, or by damage induced by physical challenges such as hypotonicity, ultra-sound, or hydrodynamic stress. We produce enlarged multinucleated leukemia cells using Cytochalasin B, which is a pharmacological agent, produced by the fungus *Helminthosporium dematioideum*, that disrupts the actin microfilaments in the cytoskeleton of cells and thereby inhibits cytokinesis. Cytochalasin B, or CB,

affects a wide variety of transport and motility processes, including cell morphology, and it inhibits cell locomotion, cytoplasmic streaming, blood clot retraction, cytokinesis, and axonal growth cone activity (Lin et al., 1978).

The destruction of actin-microfilaments inhibits cell movement. CB caps the barbed end of growing actin microfilaments causing a decrease in actin polymerization, and changes the morphology of actin filaments in any cell. It is also possible that CB inhibits movement by acting on some other cell component (Lin et al., 1973). To identify and characterize the receptors of the drug, a study was done by Lin, Santi, and Spudich, in which they developed an easy procedure for preparing [ $^3\text{H}$ ]cytochalasin B of high specific activity. The initial studies showed the binding of this [ $^3\text{H}$ ]cytochalasin B to a variety of cell types. Human neoplastic glial cells treated with medium containing  $10^{-5}$  M CB for one hour changed from the normal form to a stellate shape, but the effect was fully reversible. Within an hour after removal of CB, the glial cells returned to their normal condition (Lin et al., 1973). Cytochalasin B has rapid and reversible effects at the concentration range of 100nM and 10uM in numerous biological systems.

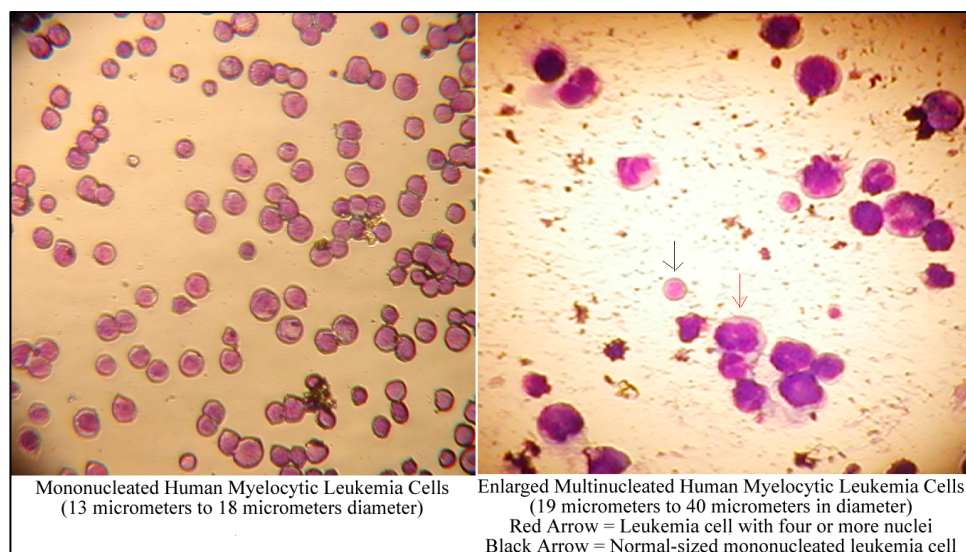
Cytokinesis is the process by which the cytoplasm is cleaved in two. It begins in anaphase but finishes only after two daughter nuclei have been formed. Cytokinesis involves the actin filaments of the contractile ring, which divide the cell in two. The cleavage furrow of the plasma membrane is formed by the action of the contractile ring underneath it. Cytochalasin B disrupts cytokinesis, by

inhibiting the formation of the contractile ring. The cleavage furrow therefore does not form and the cell does divide.

Frank O'Neill (1975) discovered that human normal and malignant cells respond to Cytochalasin B differently. Cancer cells with a disrupted actin cytoskeleton continue to produce nuclei and they become multinucleated and grossly enlarged, because they cannot complete cell division. Normal cells do not become enlarged and multinucleated when the actin cytoskeleton is damaged, but, rather, exit the cell cycle and enter a resting state until microfilament integrity is restored. Treatment of cells with CB can result in some reduction in viability, but the effects of CB are reversible, especially for normal cells (O'Neill, 1975). This reversibility of the effects of CB may allow surviving viable cells to return to their condition prior to CB treatment after the CB is no longer present. Thus surviving CB-treated cancer cells may retain the capacity to return to their pre-treatment state and retain their pathogenic potential. Asaga et al. (1983) echoed this result in work they did involving tumor cells and CB treatment. They discovered that polynuclear cells increased in all cancer cases when Cytochalasin B was applied to the cultures. They saw very little increase in polynuclear cells in benign tumor and normal mammary gland cultures (Asaga et al., 1983).

Using the observations and previous research done on Cytochalasin B, our lab is using CB on U937 human promyelocytic leukemia cells to induce multinucleation. We postulate that multinucleated cancer cells produced by treatment with CB will enter apoptosis when exposed to X-ray or to DNA-directed anti-cancer agents because they will have more nuclei, and therefore

more DNA targets: They can have four to eight or more targets as opposed to one (See Figures 2 and 3). In addition, multinucleated cells are bigger and may be more fragile and easier to kill than mononucleated cells because of that increased



**Figures 2 and 3: Mononucleated Human Myelocytic Leukemia Cells (Figure 2) and Enlarged, Multinucleated Human Myelocytic Leukemia Cells (Figure 3)**

cell volume.

U937 human promyelocytic leukemia cells are used in this work because the cells grow in suspension culture. Since they do not form aggregates or adhere to the culture flasks, they can be easily and accurately counted and sized in a Beckman-Coulter Particle Counter and in a hemocytometer. This makes it easy to determine cell size and number. We propose that the microfilament-controlled difference in cell cycle entry between neoplastic and normal cells can be exploited as an approach to therapy based on chemical and physical challenges to enlarged multinucleated cells. We propose further that normal cells that have been induced to exit the cell cycle and to enter a resting state will be protected from cell cycle

specific anti-neoplastic treatment protocols, and will not be affected by challenges based on increased cell size and multinucleation.

*Identification of Properties of Single Cells: As Populations or As Individuals*

In order to determine whether enlarged multinucleated cancer cells have increased sensitivity to damage by physical or chemical challenges, it is necessary to be able to determine quantitatively whether the enlarged cells are being selectively damaged in the presence of normal-sized mononucleated cells. It is also necessary to establish that at least some of the enlarged multinucleated cells remain viable and clonogenic and therefore remain a target to chemotherapy. We must be able to separate the enlarged multinucleated cells from the mononucleated cells as populations, or alternatively we must be able to monitor the fate of individual enlarged multinucleated cells by microscopic analysis.

Work by Gold et al. (2006), deals with physical separation of populations of enlarged multinucleated human leukemia cells from mononucleated normal-sized leukemia cells. Physical separation of large and small cells is needed to determine the differential chemical and physical response of enlarged, multinucleated cells in comparison with non-enlarged mononucleated leukemia cells. A filter system was devised using a 19 $\mu$ m nickel porated sieve to separate enlarged cells greater than 19 $\mu$ m in diameter from normal leukemia cells with diameters of 15 $\mu$ m. The cells were allowed to settle through a 3.5 inch diameter 19 $\mu$ m nickel-porated sieve with no hydrostatic head and no fluid flow through the filter. Cell distribution sizes were determined with a Coulter Counter, and the

viability was determined by hemocytometer counts, re-growth assays, and cloning assays in agarose.

Gold et al. (2006) used the purified cells separated from the sieve to test the susceptibility of the cells to damage by ultrasound. In their experiments, they sought to determine whether there was a sonic sensitivity in cells treated with cytochalasin B that can lead to cell disruption and cell death because CB-treated cells were grossly enlarged. Exposing both CB-treated and control U937 leukemia cells to ultrasound showed that at identical lengths and intensity of sonic exposure, the CB-treated cells are more damaged than the control cells. They also found that there was a statistically significant retardation in growth rate of sonicated CB-treated cells two and six days after sonication. Control cells did not show this same response to the ultrasound. In leukemia treatment, this may introduce ultrasound as a physical treatment modality. However, even in separated CB-treated U937 leukemia cells, non-enlarged cells remain at a level of 5%, and in unseparated CB-treated cells the small cells may range from 15% to 35% of the total. Thus, it becomes important to be able to distinguish the behavior of enlarged cells in the presence of non-enlarged cells.

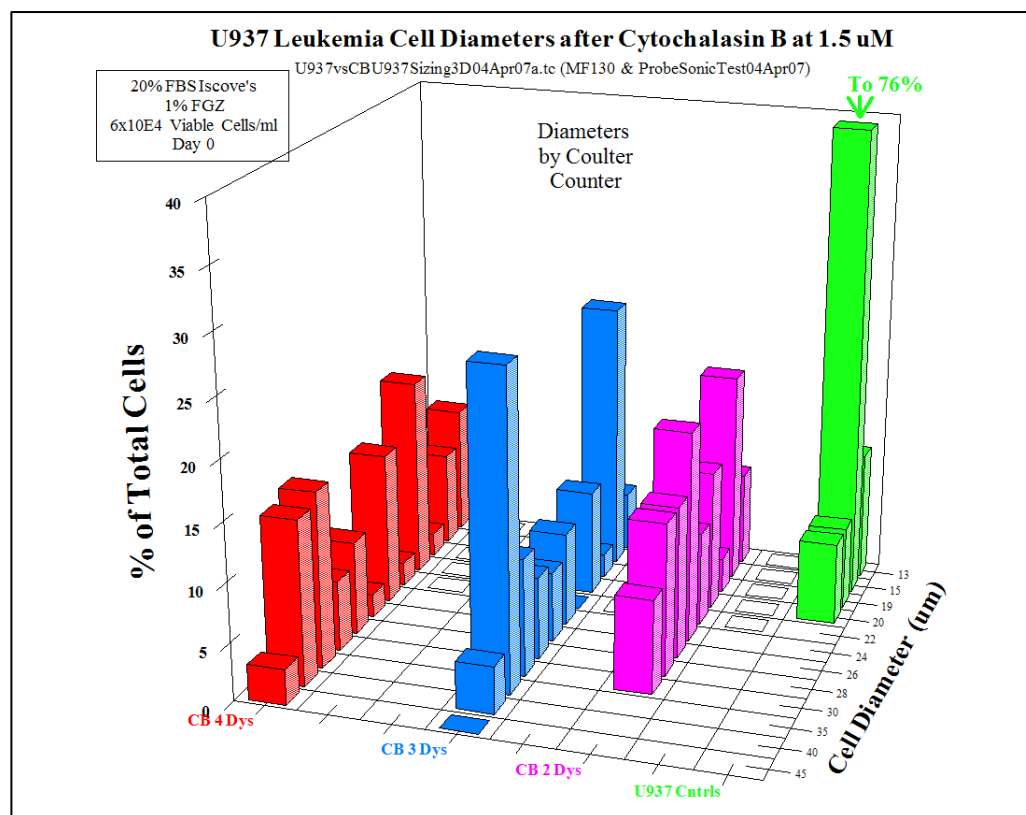
Work presented here complements work done by Gold et al. (2006), and develops methods for observing the reproductive viability of individual leukemia cells either by limiting dilution assay and growth of single isolated identified cells in suspension culture, or by cloning assay of single identifiable cells fixed in place in semi-solid medium. Monitoring the growth of individual cells in suspension culture allows us to determine by dye-reduction assay the number of viable cells



that a single cell can produce in a given time. Cloning of individual identified cells in semi-solid medium allows us to determine whether an observed cell is able to clone, and if so, the type of hematopoietic clone that the cell produces, whether undifferentiated myeloid, or partially differentiated granulocytic, monocytic, or erythroid. A clone is defined as a cell that is identical to the parent that produced it, but a clone also has to be defined by some designation of how many cells constitute it. A little clump of 8 or 16 cells is not a clone because it means that a cell divided only 4 times before stopping. Usually one defines a clone as having 50 or more cells.

Based off of preliminary studies determined by Dr. Fondy, it has been concluded that U937 cells are treated with CB under conditions that convert more than 65% of the cells to the enlarged multinucleated state (1.5um CB, 36 to 48 hours, 20% Iscove's medium). CB-treated enlarged multinucleated U937 cells retain a cloning efficiency in agarose of 9% to 15%. Since these cells contain from 15% to 35% small cells, and the cloning efficiency of untreated U937 cells is 50% to 70%, CB treatment does lower the cloning efficiency. We need to determine whether some of the enlarged multinucleated CB-treated cell remain clonogenic and, if so, what proportion of the clonogenic cells seen in the CB-treated population are enlarged and multinucleated cells. Figure 4 shows the size progression of U937 cells after CB treatment for two days, three days, and four days. Treating cells with CB causes a size shift from 13u all the way up to 45um after four days of treatment, showing the extent to which CB can enlarge cells. A

cell that is 45u in diameter is 27 times larger in volume then the original cell that is 15u in diameter.



**Figure 4: U937 Cell Diameters after CB-treatment for 2 days, 3 days, and 4 days**

In order to determine whether the enlarged multinucleated cells remain viable and potentially pathogenic by direct microscopic observation, the behavior of the enlarged cells must be measurable in the presence of normal non-enlarged leukemia cells. We must be able to look at the fate of individual cells. If the enlarged multinucleated U937 leukemia cells remain viable, clonogenic, and potentially pathogenic, the cells would be important targets for new approaches to therapy directed toward enlarged multinucleated cancer cells.

Looking at individual cells allows us to determine whether enlarged leukemia cells retain clonogenic potential and allows us also to determine the proportion of enlarged cells that retain clonogenicity. We are also able to correlate cell size with clonogenic potential to determine whether there is a relationship between the diameter of the seeded cells and their ability to clone in agarose. The types of hematopoietic cell clones that are formed in agarose are also established in order to determine whether differentiation of the U937 promyelocytic leukemia cells toward erythrocytic, granulocytic, or monocytic lineages is affected by CB-treatment.

My work specifically looks to see whether individually identified enlarged cells or small cells treated with CB are cloning. Enlarged cells are multinucleated cells and small cells are mononucleated cells. I want to determine the clonogenicity of the enlarged cells so we can see the correlation between multinucleation and cloning. It is also necessary to determine the fate of individual cells in order to determine quantitatively whether the enlarged cells are being selectively damaged in the presence of normal-sized mononucleated cells when treatments such as sonication are carried out. My work determines what proportion of the enlarged, multinucleated cells are clonogenic. The more clones there are, the more viable the cells are, and the more important they are as potential therapeutic targets.

If the enlarged multinucleated CB-treated leukemia cells retain a significant cloning efficiency, they are potential targets for therapy by chemical or physical agents. Work done by Fondy et al. (1990), demonstrated the effects of

CB *in vivo*. They found that CB administered subcutaneously at 100mg/kg delays the time of tumor appearance, inhibits tumor growth rate, and prolongs host survival in B16F10 melanoma and M109 lung carcinoma in subcutaneous tumor challenge. This shows that CB does work as a therapeutic agent in mouse cancer model systems. My work points to ways to improve the pre-clinical efficacy of CB by focusing attention on enlarged CB-treated cancer cells in the treated animal that remain potentially pathogenic and that may be susceptible to killing by other therapeutic treatments such as X-ray, DNA-directed anti-neoplastic agents, microtubule-directed agents, sonication, and other physically directed therapies.

## **Materials**

Fluoresbrite™ Plain YG 20.0 Micron Microspheres  
 BioWhittaker® Phosphate Buffered Saline (PBS)  
 U937 Human Promyelocytic Leukemia Cells  
 Atlanta Biologicals 20% Fetal Bovine Serum in Iscove's Medium  
 Cytochalasin B  
 Sigma® Trypan Blue Solution (0.4%)  
 Model Z1 Beckman-Coulter Particle Counter  
 Hemocytometer  
 Reichert Inverted Light Microscope  
 MICROTEST™ 96-Well Assay Plate, Optilux™  
 Millipore Milli Wrap  
 Falcon® 50mL Tissue Culture Flask  
 Forma Scientific Incubator  
 SeaPlaque® Agarose

## **Methods**

### *Sterilization and Sizing of 20 u Polystyrene Fluorescent Beads*

In order to determine the actual range of size distribution of the normally 20u polystyrene fluorescent beads, the beads were counted and sized using the Beckman-Coulter Particle Counter. Dr. Thomas Fondy counted the beads at  $2.2 \times 10^6$  beads/ml in water as received. Seven hundred and fifty microliters of bead suspension were autoclaved and diluted into 1.5ml sterile PBS (1:3 dilution). The beads were counted as  $3.7 \times 10^5$  beads/ml and diluted 1:4 in sterile PBS to give approximately  $1 \times 10^5$  beads/ml. Hemocytometer counts gave  $1.7 \times 10^5$  beads/ml. The Coulter counts and sizing gave  $9 \times 10^4$  beads/ml.

### *Enlarging U937 Leukemia Cells with Cytochalasin B*

U937 human promyelocytic leukemia cells were seeded at  $5.0 \times 10^4$  viable cells/ml in 20% fetal bovine serum in Iscove's medium that has 2% by

volume of 10,000 units/ml penicillin and 10mg/ml streptomycin, 0.5% gentamicin sulfate, and 2mM glutamine. The U937 cells were treated with 1.25 uM to 2.0 uM concentrations of Cytochalasin B, for 36 to 48 hours, or two cell cycles. These conditions were chosen because they virtually stop cell proliferation, allowing the cells to enlarge and multinucleate. CB-treated U937 cells retain 5% to 30% cloning efficiency depending on CB concentration, time of exposure and serum concentration. Trypan blue dye exclusion was used to determine viability. Model Z1 Beckman-Coulter Particle Counter was used to establish the size distribution and cell number of the enlarged, multinucleated cells.

#### *Cell Viability Determination*

Cell viability was determined using a trypan blue dye exclusion test and hemocytometer. The trypan blue dye test was used because it is based on the principle that viable cells do not take up the dye whereas non-viable cells do. Viable cells have an intact membrane, so they are able to exclude the dye, whereas non-viable cells lack an intact membrane and take up the trypan blue. Fifty microliters of cell suspension and 50ul of 0.4% Trypan Blue stain in isotonic saline were mixed and the mixture was transferred to each of the two hemocytometer counting chambers. Under a light microscope using a ruled eyepiece reticle or by gauging size seen by comparison with hemocytometer markings, the cells were scored as either small (<19u) or big (>19u), and for viability. The total percent viability and cell sizes were determined for the population. The full spectrum of cell sizes was determined with a Beckman

Coulter-Particle Counter. The cells ranged from 13u to 45u in diameter by reticle measurement of cells in a hemocytometer using a compound microscope or by Beckman-Coulter Particle Counter.

#### *Agarose Cloning in 96-Well Plates*

Distribution of single cells by limiting dilution seeding of wells in a 96-well plate allows one to identify and measure individual cells and to determine the fate of specifically identified cells after growth in suspension medium for twelve days. The concentration of cells and beads in the mixture used to seed the 96-well plates determines only the probability that there will be a single cell and five beads in any one well. The polystyrene sterile beads are used to help focus in the right microscopic plane and as size standards to help determine whether the cells are enlarged cells or small cells.

There are wells in which no cell is observed, and wells in which up to six and even more are occasionally observed. In cases where a single well is observed to have more than one cell at the time of seeding, if that well is observed to produce a growing suspension of cells during the cloning assay period, it is not possible to determine whether one or more cells were clonogenic and thus it is not possible to determine precisely which of the two or more cells was clonogenic. However, if the two or more cells in a single well are both (or all) enlarged cells, one can conclude that at least one enlarged cell was clonogenic.

If the positions of the two or more cells in a single well can be fixed by seeding the plates with cells and beads in 0.75% agarose medium, the positions of

single identified cells can be determined and the clonogenicity of each cell can be established. Furthermore, it is difficult to establish in suspension culture what hematopoietic cell lineage was generated by any clonogenic cell, whereas in agarose cloning in culture flasks or 96-well plates this is possible. Growth of U937 leukemia cells in agarose semi-solid medium in test tubes also allows one to establish the type of hematopoietic clone that is produced by observing the physical characteristics of the clones formed. However, in test tubes it is not possible to determine at the time the agarose tubes are seeded with cells the size of the cell that produces a clone after twelve days. In order to combine the advantages of growth in individual wells with the ability to identify a final clonal lineage, the methods of seeding in agarose suspension medium and cloning in culture flasks and 96-well plates were combined.

#### *CB-Treated Cells Seeded in 96-Well Plates in Agarose with Centrifugation*

When seeding 96-well plates with beads and cells in agarose, the beads and cells are not always in the same microscopic plane. This experiment spins the plate in a Beckman centrifuge to see if all contents in the agarose will settle to the bottom plane.

Ten milliliters of 1.25uM CB Treated U937 cells in 20% FBS at  $3 \times 10^4$  viable cells/ml were prepared. In addition, a preparation of agarose was made with 600 cells/ml and 600 beads/ml, which equals 9 cells and 9 beads per 15ul. Twenty wells of the inner 60 wells (Rows B and C) of an optically clear plate were seeded with 15ul of mixture and allowed to congeal (Rows A and H, the end



rows, and columns 1 and 12, the end columns, were not seeded with cells but rather were kept empty to allow us to place sterile water and 1% Amphotericin B in them to restrict evaporation from the inner 60 wells and trap mold spores entering from the outer edges of the plate).

Another preparation of agarose was made with 50 cells/ml and 50 beads/ml, which equals 10 cells and 10 beads per 200ul. Another 20 wells on the inner 60 wells (Rows C and E) of the optically clear plate were seeded with 200ul of mixture each (10 cells and 10 beads per well). The plate was immediately spun down in the centrifuge to get the cells and beads in Rows B,C,D and E to settle on the bottom of the wells. After centrifugation of the plate, another preparation of agarose was made with 9 cells and 9 beads per 15ul. The remaining 20 inner wells (Rows F and G) were seeded with 15ul of contents each and allowed to congeal.

Two hundred microliters of agarose medium were added into each of the wells in rows B,C,F and G to bring these volumes to 215ul. The agarose was allowed to congeal and then some of the 60 wells were photographed by inverting the plate and viewing the cells near the optically clear bottom. Two hundred microliters of sterile water with Fungizone were added to the outer 36 wells (Rows A and H and Columns 1 and 12) and the plate was covered with a gas permeable membrane. The plate was incubated and allowed to clone for two weeks. At the end of two weeks, the plate was scored for wells that cloned in reference to the positions of the cells and beads previously identified and photographed.

*Agarose Cloning in 12.5 cm<sup>2</sup> Flasks:*

In agarose, cells will not move when cloning. Therefore, it is possible to see exactly which cells cloned. Cells seeded in agarose medium in 12.5 cm<sup>2</sup> culture flasks in a thin layer of agarose become fixed in a single focal plane immediately above the optically clear plane of the flasks after the agarose congeals. At that point, the size and position of the seeded cells can be determined and recorded. At the end of the cloning period the position and hematopoietic cell lineage of any clone was traced back to whatever cell was recorded in that position at the time of agarose cloning. The daughter cells produced by each clonogenic cell will remain at the sites of the stem cell because the cells cannot move in semi-solid medium. This is the advantage of cloning in agarose over cloning in liquid medium. In liquid medium, one must physically isolate potentially clonogenic cells from one another by limiting dilution plating in microtiter wells or by Live Cell Array slides in order to be able to determine the properties of individual source stem cells.

Each experiment used three tissue culture flasks. Two of these flasks received 35ul of CB cells and 50ul of beads (500 beads) in 1ml of agarose. After the cells and beads congealed in this layer, 3ml of agarose medium are added to each flask and placed in the carefully humidified incubator at 37°C for one day to allow the agarose to congeal. If the agarose is not completely congealed, it will slide off the edges of the culture flask when inverted, making it impossible to score and record the contents.

Day 1 after seeding, the CB-flasks were inverted under the Reichert Microscope. Six fields that were especially rich in big and small cells were selected per flask. The center of each of these fields was marked with a different colored sharpie marker. Using the stage micrometer scale on the microscope, the position of each colored mark was recorded and then the pattern of cells and beads in each field were recorded using a circular polar graph paper. The flasks were allowed to clone in the incubator at 37° C for two weeks. After two weeks, the flasks were observed under the microscope at their labeled colored marks. Positions of the clones were traced to the cells originally in the flasks at the positions recorded visually at the time of well-seeding.

#### *Limiting Dilution Assays in 96-Well Plates*

Limiting dilution assays in 96-well plates allow us to clone cells in liquid medium. Using these methods, one can physically isolate potentially clonogenic cells in order to determine the properties of individual source stem cells.

Enlarged multinucleated U937 cells were diluted to 300 cells/ml in 20% FBS Iscove's medium. Twenty micron sterile fluorescent beads were added to this suspension at 1000 beads/ml. Five microliters of this mixture were carefully placed under sterile conditions into the center of each well in the 96-well optically clear bottomed plates, using a Hamilton syringe injector or a 10ul micropipettor. This 5ul suspension gave an average of approximately 1.5 cells and 5 beads per well. The plate was inverted under a Reichert Microscope. The 5ul sample remained attached to the inverted optically clear bottom and the well contents

were viewed and scored at 56X. Each well was examined and its bead and cell contents were recorded on a 96-well scoring sheet. As in the agarose cloning, the beads help to locate and focus on the bottom of the wells, and to estimate the sizes of cells in comparison with the 20u beads.

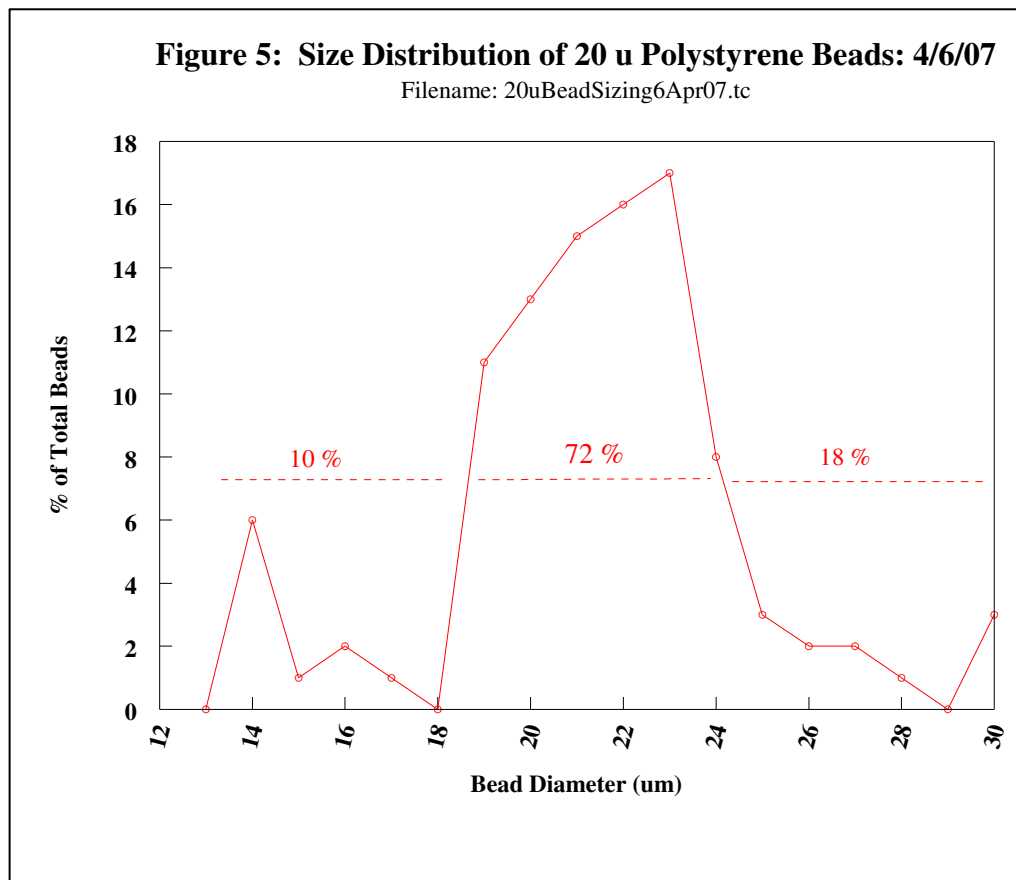
Cells were scored as either “BC” for “big cell,” which is a cell greater than 19u, or “SC” for “small cell,” which is a cell smaller than 19u. Big cells are enlarged and multinucleated and small cells are small and mononucleated. After scoring the contents of each well, 195 microliters of 20% FBS Iscove’s medium was added to each well, using an 8 track pipettor. The wells were covered with a sterile gas-permeable membrane to reduce evaporation and to prevent mold spores from entering the well.

Typically, three plates were scored for each experiment. Plate one was scored, growth medium was added, and it was incubated before plate two was started. If the cells are left out of medium for too long, they will die, which is why it is important to load one plate, score that plate, and add the growth medium before starting another plate. The plates were incubated in a carefully humidified incubator at 37°C for 12 days, at which point the wells were scored for clonogenicity. The plates were followed for an additional three or four days to score weakly clonogenic plates.

## **Results**

### *Sizing of 20 u Polystyrene Fluorescent Beads*

After counting and sizing the normally 20u diameter polystyrene fluorescent beads with a Beckman-Coulter Particle Counter, it was found that 10% of the beads are 13u to 18u in size, 72% of the beads are 19u to 23u in size, and 18% are 24u to 30u in size. Figure 5 shows the distribution of these bead size counts. The modal peak is seen around the bead diameter of 23u. The nominal bead size is designated as 20u, but the range of almost equivalent bead frequencies is from 20u to 24u (13 to 17% for each of these diameters), with the 19u and 24u beads each being 10% of the total.



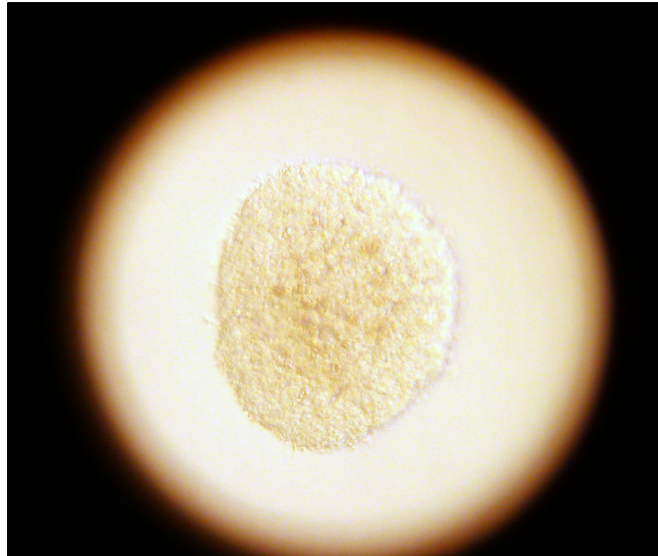
Ninety percent of the beads are 19u or greater in diameter. When used as an index of cell size for cells co-seeded with beads, the size distribution shown in Figure 5 allows us to determine that any cell that is equal to or larger than the beads when several beads are viewable for comparison must be 19u or larger in diameter and thus is an enlarged cell by our criteria.

### *Agarose Cloning in Flasks*

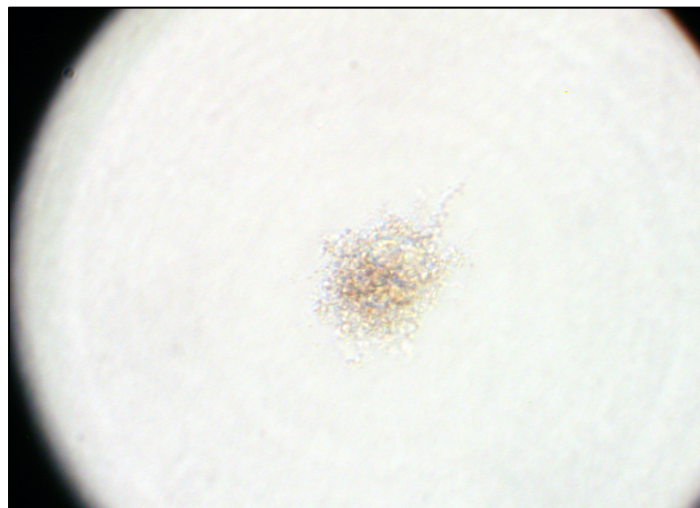
Our purpose for seeding the cells in flasks in agarose is to allow us to determine the positions of large and small cells after seeding so that if a clone appears at a given position in a flask, the size of the source stem cell can be established. The daughter cells produced by each clonogenic cell will remain at the site of the stem cell because the cells cannot move in semi-solid medium. This is the advantage of cloning in agarose over cloning in liquid medium. In liquid medium, one must physically isolate potentially clonogenic cells from one another by limiting dilution plating in microtiter wells or in Live Cell Array Slides in order to be able to determine the properties of individual source stem cells.

I originally tested to see if the cells would clone in agarose when plated in a thin layer of agarose in culture flasks. The seeded cells had to be in a thin coplanar layer next to the optically viewable surface of the 96-well plate in order to be able to see the individual cells and to determine their sizes and record their positions. Colonies of differentiated cells formed in the agarose (See Figures 6 and 7). Using images from Stem Cell Technologies manual for comparison, I

concluded that a portion of the differentiated colonies are burst forming unit erythroid colonies and the remainder are colony forming unit granulocyte macrophages.



**Figure 6: A large burst-forming unit-erythroid differentiated colony that formed in agarose**



**Figure 7: A colony forming unit granulocyte, macrophage differentiated clonal colony that formed in agarose**

In the experiments done cloning CB-treated cells in agarose, a total of 278 cells were observed in 22 microscopic fields. The location of each field was marked by a different colored marker on the culture flask and, using a circular eyepiece reticle, a “map” of cell and bead position was sketched on polar graph paper. Of these fields, three and a half fields had to be discarded because the entire field, or half of one field, was covered in clones by the end of two weeks, making it impossible to determine which cells individually cloned.

Excluding the cells from these three and a half fields gives me a new total of 218 cells in 18.5 microscopic fields. Table 1 shows the breakdown of enlarged and small cells. I was working with a population of greater than 80% enlarged cells because these cells were previously separated by 19u nickel-porated filtration. Therefore, I was mainly working with cells greater than 19u. The overall cloning efficiency for these experiments in agarose and the cloning efficiency of the enlarged cells are unusually high, but using this method, we probably are able to see clones that we would otherwise not see in test tubes.

<b>Cells</b>	<b>How Many</b>	<b>Percentage of Population</b>	<b>Cells that Cloned</b>	<b>Cloning Efficiency</b>
<b>Total</b>	218	100%	82	38%
<b>Enlarged</b>	208	95%	82	39%
<b>Small</b>	10	5%	0	0%

**Table 1: Results of Cloning CB-treated U937 Cells in Agarose in Culture Flasks**



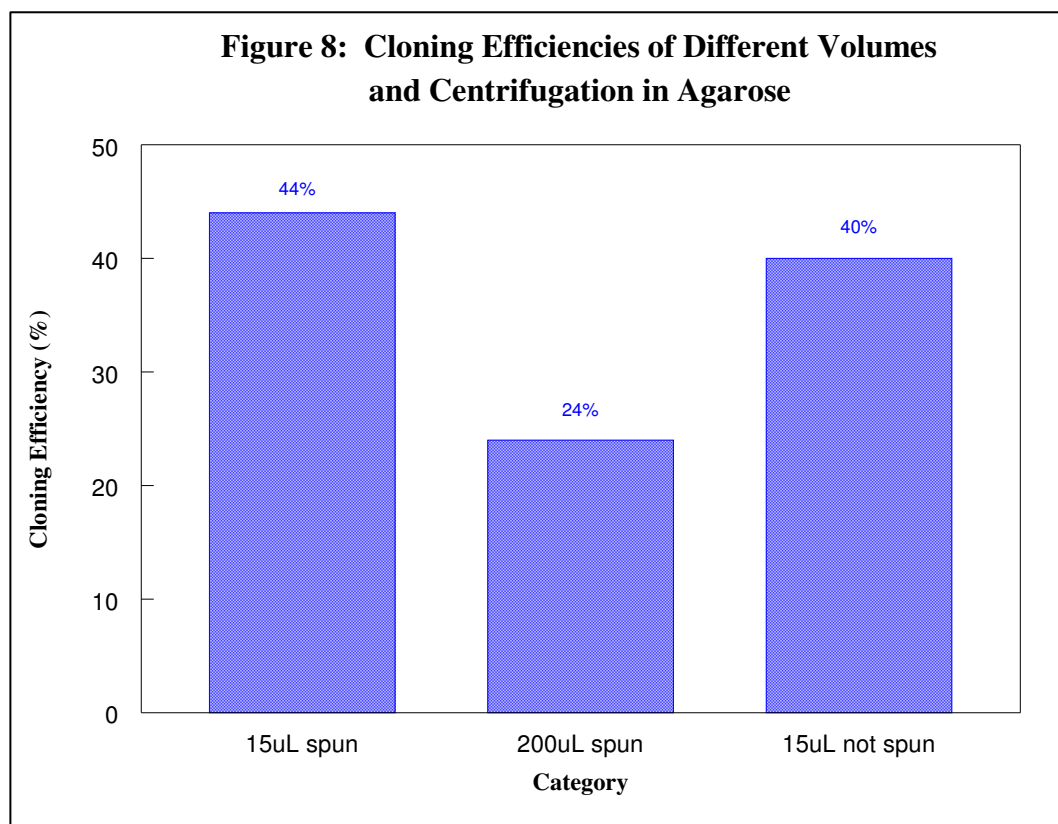
*CB-Treated Cells Seeded in 96-Well Plates in 15ul Agarose vs. 200ul Agarose with Centrifugation*

When seeding the cells in agarose, the beads and cells end up in different planes, unless the beads and cells are seeded initially in a thin layer which is overlaid with agarose medium after the initial thin layer has congealed. Thin layer seeding and agarose overlaying are difficult to do in 96-well plates. For this reason, I performed this experiment to find a way to get the beads and cells in the same microscopic plane in 96-well plates. Table 2 shows how the plate was divided and loaded.

<b>Rows</b>	<b>Volume</b>	<b>Centrifuge</b>	<b>Number of Wells</b>	<b>Number of cells</b>	<b>Number of Clones</b>	<b>Cloning Efficiency</b>
<b>B and C</b>	15ul	Yes	20	100	44	44%
<b>D and E</b>	200ul	Yes	20	100	24	24%
<b>F and G</b>	15ul	No	20	90	36	40%

**Table 2: Experiment and Results of Varying Volume with Centrifugation and Effects of Centrifugation**

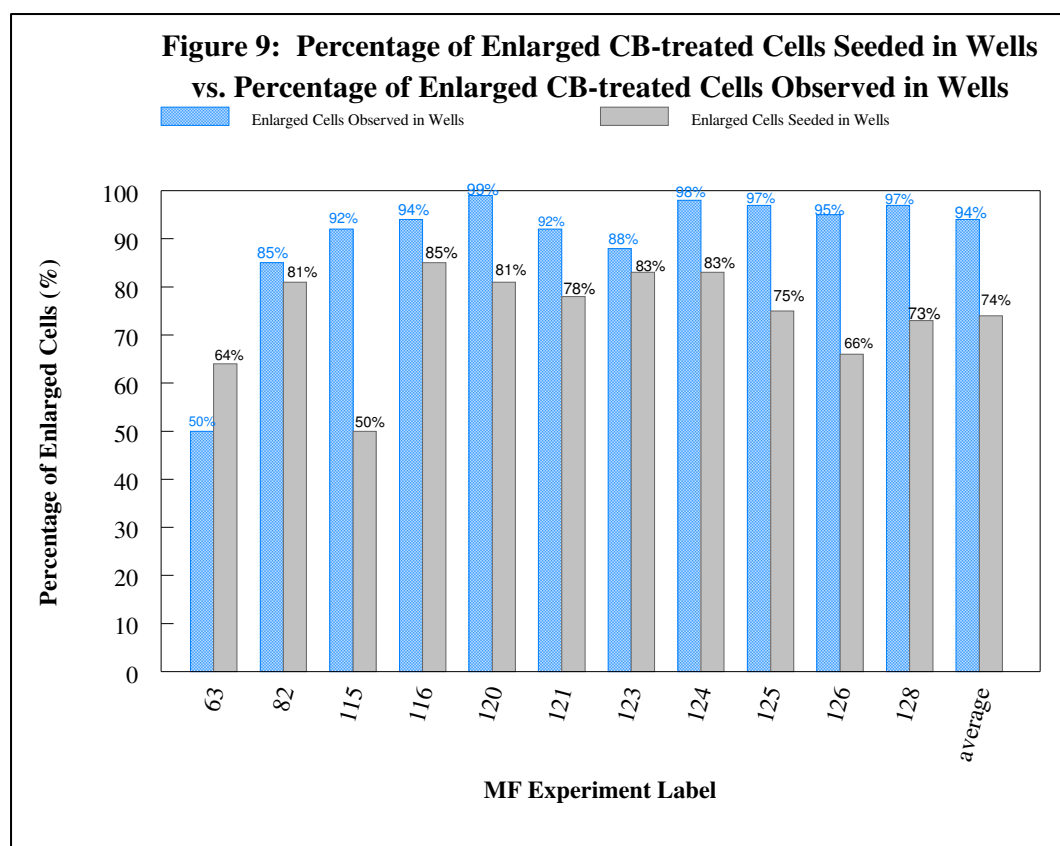
The cells in this experiment were not originally recorded as being enlarged or small because the purpose of this experiment was to show the effects of centrifugation and volume of agarose seeded on cloning efficiency. Table 1 and Figure 8 show the cloning efficiencies for the different volumes and with and without centrifugation.



I determined that a lower volume of cells and agarose spun down is ideal for getting the beads and cells in a single focal plane for viewing and mapping of sizes and positions. The cloning efficiency of the wells with 15ul spun down and the wells with 15ul not spun down was very similar (44% compared to 40%), so centrifuging the wells does not affect clonogenicity. Thus, centrifuging puts the beads and cells in the same plane, which helps to score the contents, and it does not affect the cloning efficiency. Seeding the cells in 200ul of agarose and attempting to centrifuge the cells and beads to the bottom of the wells did not work as well in terms of cloning efficiency as did seeding in 15ul (24% compared to 44%), even though I saw 100 cells in both cases.

*Limiting Dilution Assays in 96-Well Plates: Percentages of Enlarged Cells Observed in Wells vs. Percentages of Enlarged Cells in Seeded in Suspension*

The results of eleven limiting dilution assay experiments will be incorporated into the following sections. In each of these experiments, I determined the percentage of enlarged CB-treated cells that I observed in the wells by dividing the total number of enlarged cells I recorded seeing in the wells by the total number of cells seeded. Before I seeded the wells, Dr. Fondy determined the percentage of enlarged cells that were in the suspension by Beckman-Coulter particle counting. Figure 9 shows the comparison between the percentage of enlarged cells that I actually observed in the wells and the percentage of enlarged cells that Dr. Fondy determined were in suspension.



My data indicates that I seeded a higher proportion of enlarged CB-treated cells in the wells than what Dr. Fondy originally recorded and so I was therefore working with a higher population of enlarged cells. This discrepancy means that the enlarged and small CB-treated cells were not loaded into the wells or observed in the wells in equal proportions either because I was able to see enlarged cells more easily by careful microscopic examination or because some small cells may be broken up during the seeding operation causing them to be unseen by microscopic examination. If this latter alternative is correct, the missing small cells must be damaged and non-viable. I was working with 94% enlarged cells CB-treated cells and 6% small CB-treated cells. To determine the average of enlarged and small cells, I divided the total number of enlarged cells observed in the wells by the total number of cells observed in the wells. To determine the average percentage of small cells, I divided the total number of small cells observed in the wells by the total number of cells observed in the wells.

*Limiting Dilution Assays in 96-Well Plates: Contents and Cloning Efficiencies of Total Cells*

Ideally, I aimed to seed one cell per well. Probabilities dictate that there would be some wells with more than one cell and some with no cells. Some wells contained only one cell, other wells contained anywhere from 2 to 5 cells, and other wells contained no cells (see Appendix A). In wells that contained multiple cells, I could still draw conclusions if all the cells in the well were enlarged cells or, alternatively, if all the cells in the well were small cells. The difficulty lies in

wells that contained mixtures of enlarged and small cells. If these wells clone, it is impossible to determine which size cell was responsible for the cloning, making it difficult to draw any conclusions from these wells. For this reason, a total of 68 wells had to be excluded because they contained a mixture of enlarged and small cells (In the last section of the results, I will discuss the cloning efficiencies of enlarged and small cells, including the wells that contained mixed contents).

Table 3 shows the total number of wells scored and the total number of wells that cloned for all experiments, excluding wells that had mixed large and small cells.

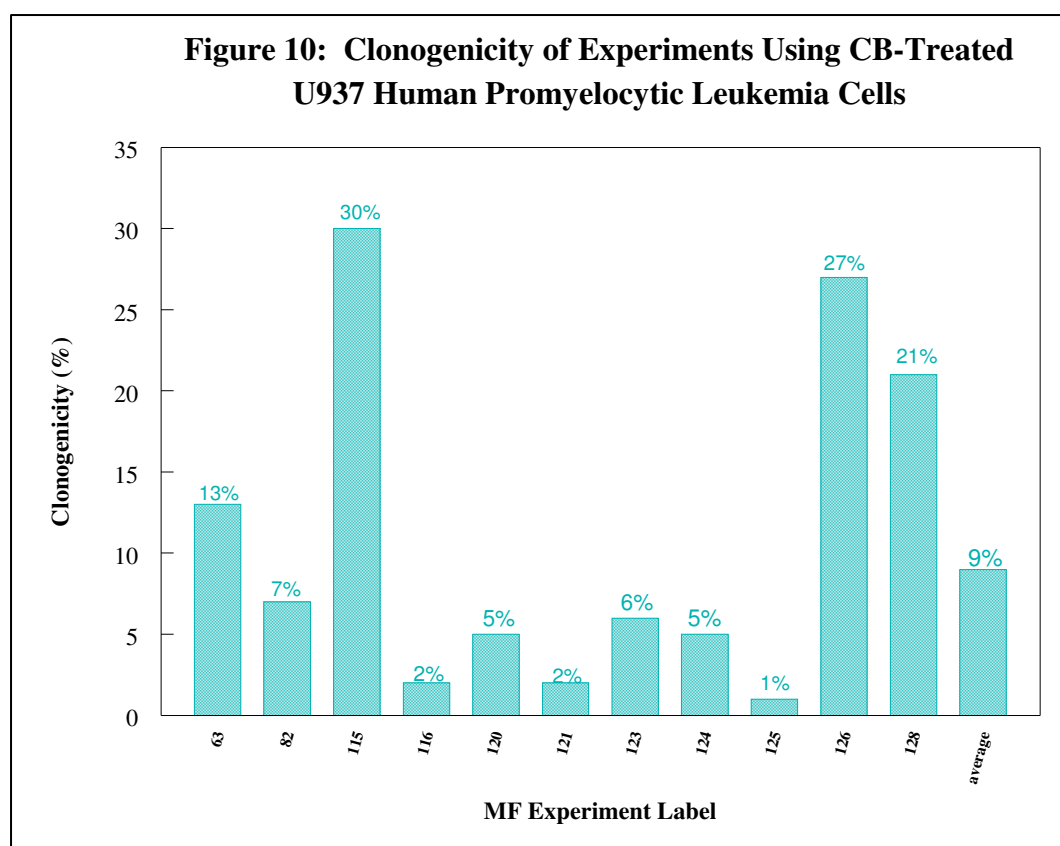
<b>Number of Plates</b>	<b>Number of Wells</b>	<b>Number of Wells that Cloned</b>	<b>Cloning Efficiency</b>
16	1,131	100	8.84%

**Table 3: Number of Wells Scored and the Average Cloning Efficiency of all the Experiments**

The total number of wells is not a multiple of 96 because wells occasionally had to be excluded for mold growth or because the loading sample hit the edge of the well. When the loading aliquot touches the edge of the wells rather than remaining as a circular dot in the middle of the wells, it is not possible to be certain of whether there are cells that remain unobserved at the interface between the sample aliquot and the walls of the wells.

I determined the cloning efficiency, or clonogenicity, for each experiment by dividing the total number of wells that cloned by the total number of wells that were scored. The results and specific cloning efficiency for each experiment and

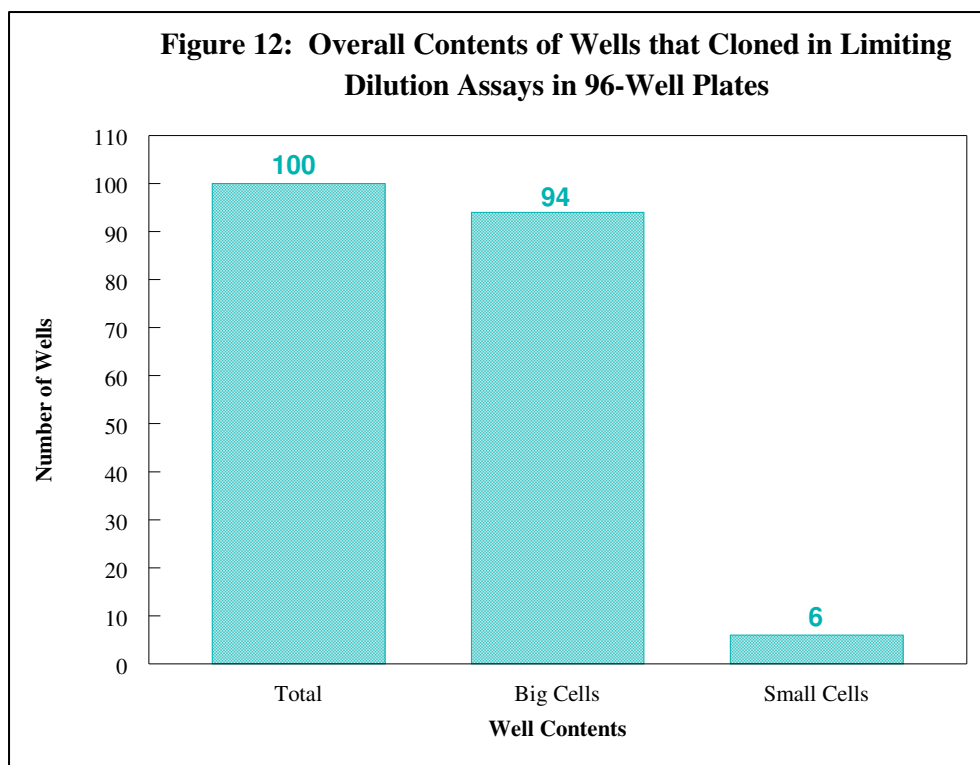
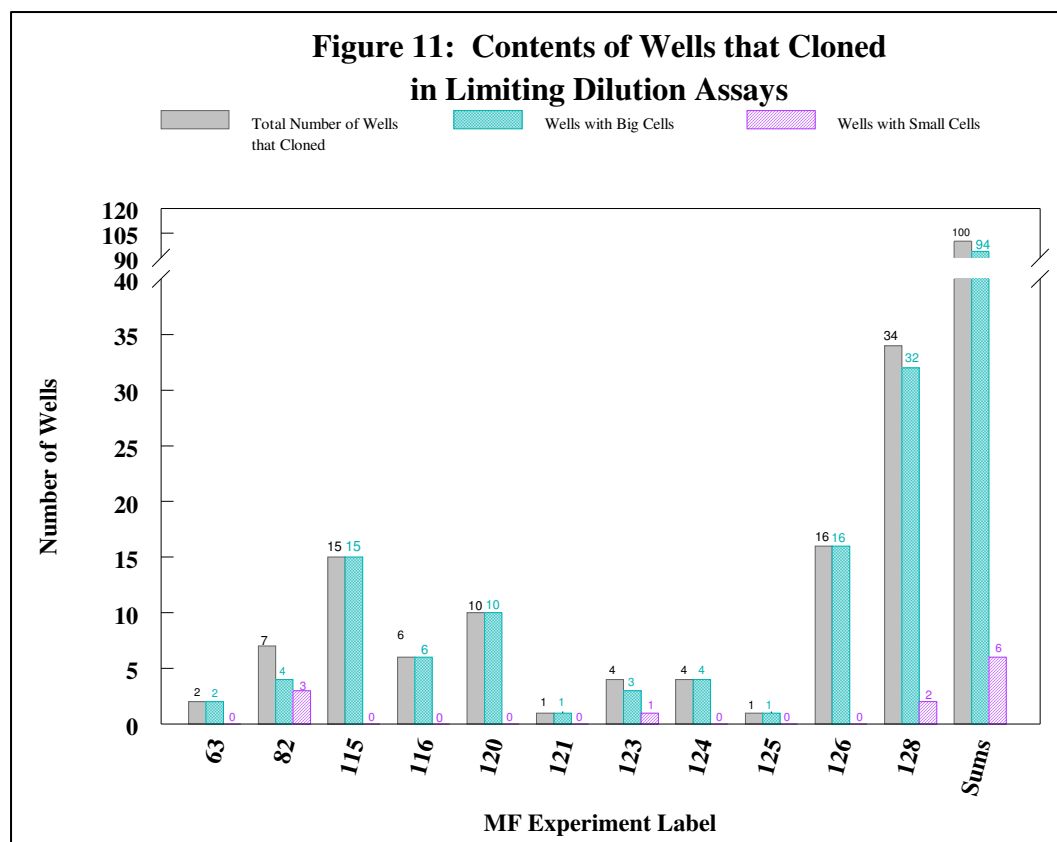
the overall clonogenicity are shown in Figure 10. The average cloning efficiency was found by dividing the total number of wells that cloned by the total number of wells that were properly seeded, in this case 100 wells with clones divided by 1,131 wells properly seeded. The average cloning efficiency was 9%. This graph shows the variation in cloning efficiencies for each experiment. I will suggest some possible reasons for these variations in my discussion section.



#### *Limiting Dilution Assays in 96-Wells Plates: Contents of Wells that Cloned*

In the 100 wells that cloned, 94 contained only enlarged cells. This means that 94% of the wells that cloned contained only big cells, thus demonstrating that the enlarged cells are clonogenic. The remaining 6 wells that that cloned contained only small cells. Figure 11 shows the contents of the wells that cloned

for each experiment. Figure 12 shows the overall contents of the wells that cloned.



*Limiting Dilution Assays in 96-Well Plates: Lower Limit Cloning Efficiency of Enlarged, Multinucleated CB-Treated U937 Cells*

Of the 94 wells containing only enlarged cells that cloned, 33 contained only one enlarged cell and the remaining 61 wells contained 2 or more enlarged cells. Thus, we know that at least 94 big cells cloned out of 2,127 big cells in 1,131 wells, giving a lower limit cloning efficiency of the enlarged cells of 4.4% (see Table 4). It is possible that in some wells with two or more big cells, more than one big cell cloned. However, I am able to establish with certainty that at least 4.4% of the CB-treated enlarged U937 leukemia cells remain viable and potentially pathogenic after CB treatment. If these viable, enlarged cells prove to be super clonogenic, meaning they produce more than 2 daughter cells because they are multinucleated, then they could be extremely pathogenic and strong targets for destruction.

*Limiting Dilution Assays in 96-Well Plates: Upper Limit Cloning Efficiency of Enlarged, Multinucleated CB-Treated U937 Cells*

If all the enlarged cells in each of the 61 multiply-loaded wells with big cells cloned, then a maximum of 224 enlarged cells cloned out of the 2,127 enlarged cells seeded. This would give an upper limit cloning efficiency of enlarged cells of 10.5% (see Table 4). There is a very low chance that more than 2 enlarged cells per well cloned. However, this means that the overall cloning efficiency range of enlarged, multinucleated U937 cells is 4.4% to 10.5%. Whether 4% or 10% of the enlarged CB-treated cells remain viable, the important



point to remember is that some of the enlarged cells remain viable and pathogenic after CB-treatment and thus are targets for therapeutic destruction.

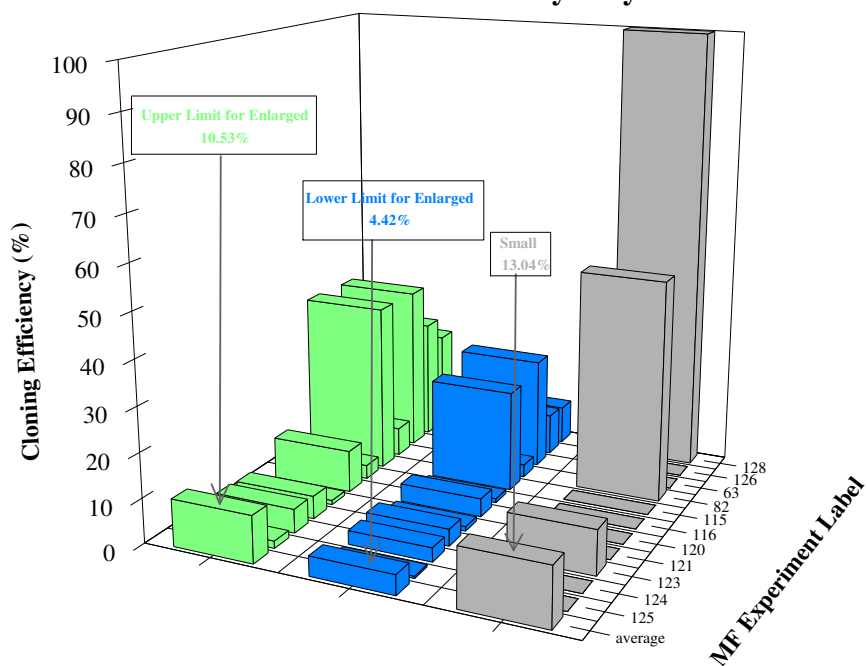
*Limiting Dilution Assays: Cloning Efficiency of Small U937 Cells in CB-Treated Populations*

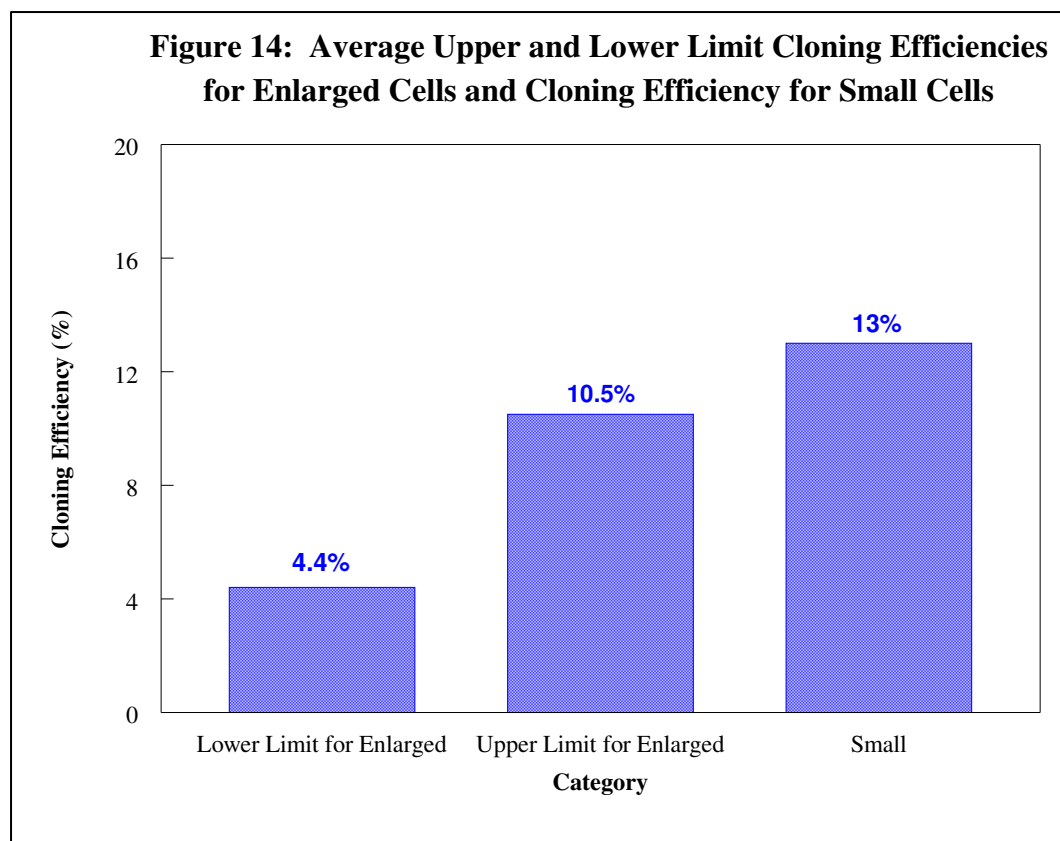
In the wells containing only small cells that cloned, only one small cell was observed in each well, meaning that an upper and lower limit cloning efficiency does not need to be determined for the small cells because a range does not need to be established. Thus, in the six wells that cloned containing only small cells, a total of 6 small cells cloned. Of the 46 small cells observed in these experiments, the 6 that cloned gives a 13% cloning efficiency for the small, mononucleated cells (see Table 4). This percentage is significantly lower than the cloning efficiency of untreated U937 mononucleated leukemia cells, which is 50% to 70%. This means that CB treatment is affecting these mononucleated cells and they are not simply U937 myeloblastic leukemia cells representative of the original population of cells treated with CB. Figures 13 and 14 show a graph distribution of the upper and lower limit cloning efficiencies for the enlarged cells and the cloning efficiency of the small cells. Figure 13 shows a 3D graph of all the experiments and Figure 14 shows the overall cloning efficiencies.

Case	Number of Wells	Number of Cells Cloned	Number of Cells Seeded	Cloning Efficiency
<b>Lower Limit for Enlarged</b>	94	94 Big	2,127 Big	4.4%
<b>Upper Limit for Enlarged</b>	94	224 Big	2,127 Big	10.5%
<b>Small</b>	6	6 Small	46 Small	13%

**Table 4: Upper and Lower Limit Cloning Efficiencies of Enlarged Cells and Cloning Efficiency of Small Cells**

**Figure 13: Upper and Lower Limit Cloning Efficiencies of Enlarged and Small CB-Treated U937 Human Promyelocytic Leukemia Cells**





*Limiting Dilution Assays in 96-Well Plates: Cloning Efficiencies for Enlarged and Small Cells if Wells That Had Mixtures of Enlarged and Small Cells are Included*

After scoring the wells, I discovered that 68 wells contained a mix of enlarged and small cells. In the previous calculations determining the cloning efficiency of enlarged and small cells, I did not take these wells into account. Thirteen of these 68 wells that contained mixes of enlarged and small cells ended up cloning, but I cannot conclude which size cells in these wells was responsible for the cloning.

If I include all the contents of these mixed wells, I end up with a total of 2,283 enlarged cells and 140 small cells seeded in the wells. I end up with

different cloning efficiencies then for the enlarged and small cells. Table 5 shows these different cloning efficiencies.

Case	Number of Wells	Number of Cells Cloned	Number of Cells Seeded	Cloning Efficiency
<b>Lower Limit for Enlarged</b>	94	94 Big	2,283 Big	4.1%
<b>Upper Limit for Enlarged</b>	94	224 Big	2,283 Big	9.8%
<b>Small</b>	6	6 Small	140 Small	4.3%

**Table 5: Upper and Lower Limit Cloning Efficiencies of Enlarged Cells and Cloning Efficiency of Small Cells Including Cells in Mixed Wells**

The cloning efficiency is barely affected for the enlarged cells; however the cloning efficiency of the small cells is quite different. Far fewer small cells were seeded in these experiments and the probability of having a small cell seeded in the same well as an enlarged cell was very likely. Ninety-four of the small cells observed were co-seeded in the same well as an enlarged cell. Only 46 small cells were in wells by themselves. Therefore, when I eliminate the wells containing mixtures of enlarged and small cells, I am eliminating the majority of the small cells.

Not only were fewer small cells seeded, but when they were in wells with mixed contents, they would sometimes be seen in clusters. The mixed wells therefore had anywhere from one small cell to four small cells, if a cluster was formed. By seeding fewer small cells, if one small cell ends up cloning, it affects the overall cloning efficiency much more than if one enlarged cell cloned. This section was included to show that there were more small cells observed in the

wells, they were just in wells with enlarged cells and I cannot draw definite conclusions from these wells.

## **Discussion and Conclusion**

### *Sizing of 20 $\mu$ Polystyrene Fluorescent Beads*

In determining the size of the polystyrene fluorescent beads, I confirm that they are sufficient to use as a size standard to determine an enlarged cell. Ninety percent of the beads are greater than 19 $\mu$ , and anything over 19 $\mu$  is considered an enlarged cell. Since the average bead size is actually 23 $\mu$ , cells that are equal to or larger than the average beads visible in the microscope field are well over 19 $\mu$  in diameter. When determining a “small cell” (a cell less than 19 $\mu$  in diameter), however, it is not accurate to say that any cell smaller than any bead is a small cell. Since I seeded anywhere from three to five, and sometimes more, beads per well, I would look at the smallest bead in the field to determine a small cell. If a cell was significantly smaller than that smallest bead, I would record it as a small cell. Furthermore, after looking at thousands of cells under the microscope, my eyes have a very accurate view of what a small cell looks like and what an enlarged cell looks like.

Overall, the beads are a helpful contributor to determining cell size and they are essential for locating the focal plane containing the cells and for focusing the microscope fields. The beads are also valuable size standards to use for comparison of cells in photomicrographs done at different camera zoom settings.

### *Agarose Cloning of CB-Treated Cells in Culture Flasks*

Although the majority of my work did not focus on agarose cloning, I can still make many valuable conclusions from this work. It has already been

established from previous work by Dr. Fondy that untreated U937 cells have a cloning efficiency of 50% to 70% when cloned in agarose in test tubes. I was not sure if my methods for cloning CB-treated cells in agarose would be successful. However, I was able to establish that CB-treated U937 human myelocytic leukemia cells do clone in a thin layer of agarose in culture flasks and they do clone in agarose in 96-well plates. This was important to determine because now these methods can be used to monitor the fate of individual cells in agarose by close microscopic examination. Examining contents under a microscope allows one to have a closer inspection and see contents that may not have been visible in the agarose cloning in test tubes.

CB-treated U937 human promyelocytic leukemia cells form differentiated colonies in thin layer agarose and in agarose in 96-well plates. I determined that these colonies are large burst-forming unit-erythroid and colony forming unit granulocyte macrophage colonies. Any type of cloning or differentiation seen indicates that the cells are still viable and are therefore potential targets for destruction. It may be that CB induces differentiation in U937 human myelocytic leukemia cells in agarose, since the untreated U937 cells that cloned in agarose in test tubes did not clone into differentiated colonies. The CB treatment may be what is causing the cells to form burst-forming unit-erythroid differentiated colonies and colony forming unit granulocyte macrophage differentiated colonies. We need to look at untreated U937 cells by these methods to see if CB is what induces this differentiation.

Cloning in agarose successfully restricts the cells to one place, unlike cloning in liquid medium. Both the overall cloning efficiency of enlarged and small cells combined in agarose (38%) and the separate cloning efficiency of enlarged cells alone in agarose (39%) are unusually high. This may be because one sees clones by this method that may not be seen in the test tubes because, as previously mentioned, the agarose flask cloning procedures view the clones under the microscope. I count as clones collections of cells that have 5u or more cells. Some of these may escape observations in test tubes. More experiments need to be run to compare the cloning efficiencies of the agarose flasks to the cloning efficiencies of the agarose tubes. This would help to explain the high cloning efficiencies seen for the CB-treated cells in the flask cloning procedure and also help to conclude if one method of cloning actually causes higher clonogenicity.

The fact that the CB-treated enlarged cells have a cloning efficiency of 39% in agarose is a very significant finding. This means that the enlarged cells are still viable and therefore potential targets for destruction after CB treatment. The small cells had a cloning efficiency of 0%, but only 10 small cells were seeded in the culture flasks, which is too few to make any definite conclusions. More CB-treated small cells would need to be seeded and cloned in the agarose flasks to see if they truly are not clonogenic.



*CB-Treated Cells seeded in 96-Well Plates in 15ul Agarose vs 200ul Agarose with Centrifugation*

This experiment was done to see if centrifuging the cells affects clonogenicity. When seeding cells in agarose, they end up in multiple microscopic planes. This makes it very difficult to accurately determine all the contents in agarose, because it is very easy to miss contents that are in multiple focal planes. Centrifugation should cause all contents in the agarose to go to the bottom of the well, making it much easier to visualize the contents and placing everything in the same plane. I needed to determine what initial volume of cells would be ideal for centrifuging the cells and beads down into a single plane, and also whether centrifuging affected clonogenicity.

In this experiment, I determined that an initial volume of 15ul of cells and agarose is preferable to an initial volume of 200ul of cells and agarose for centrifuging and for looking at the beads and clones. The cloning efficiencies were very different, 44% and 24%, respectively. The cloning efficiency between the wells with 15ul spun down was 44% and the cloning efficiency of the well with 15ul not spun down was 40%. These cloning efficiencies are very similar, so centrifuging the wells does not appear to affect clonogenicity. Centrifuging puts the beads and cells in the same plane, which makes scoring the contents of the wells both easier and more accurate, and it does not affect the cloning efficiency.

*Limiting Dilution Assays in 96-Well Plates: Percentages of Enlarged CB-Treated Cells Observed in Wells vs. Percentages of Enlarged CB-Treated Cells Seeded in Wells*

The bulk of my research focused on limiting dilution assays in 96-well plates. For each experiment, I determined the proportion of enlarged CB-treated cells I observed in the wells. Before giving me the cells to work with, Dr. Fondy determined the proportion of enlarged CB-treated cells in the cell suspension that I used to seed the wells. My results (see Figure 9) indicate that I observed a higher proportion of enlarged cells in the wells than what Dr. Fondy originally recorded. This discrepancy may have arisen because I was able to see more of the enlarged cells than the small cells by careful microscopic examination. The lower size limit of small cells for untreated U937 leukemia cells is 13u and we record “small cells” measured with the Beckman-Coulter Counter as those cells that range between 13u and 18u in diameter. It is possible that some cells as small as 13u that are detected with the Beckman-Coulter Counter and are recorded as “small cells” in the population of cells used to seed the plates, may escape visual observation in the wells.

Overall, I observed a population of CB-treated cells in the wells that were 94% large cells and 6 % small cells. The proportion of large vs. small cells used to seed the plates was actually 74%:36%. My research was specifically focused on determining whether the enlarged CB-treated U937 leukemia cells remain viable and clonogenic. The disproportionately high percentage of enlarged cells

seen in the wells does not affect the conclusion that they retain a very significant cloning efficiency of approximately 4.5%.

The disproportionately low percentage of small cells observed in the wells may mean that some clonogenicity attributed to enlarged cells could arise from unseen small cells. However, in cases where small cells were observed separate from large cells, I was able to determine that the small cells have a very low cloning efficiency, so that a few small cells that may escape observation could not produce the cloning efficiency of 4.5% seen for the 94% of the cells that are large. The fact that I observed differentiated colony-forming units in the CB-treated cells cloned in agarose indicates that CB may be inducing differentiation. Thus some or even all of the small cells may in fact be differentiated granulocytes and thus would not be clonogenic stem cells.

#### *Limiting Dilution Assays in 96-Well Plates: Contents and Cloning Efficiencies of Total CB-Treated Cells*

The variability in clonogenicity for the experiments may be related to the slight differences in procedures between experiments. Experiments MF63, MF82, MF115, MF126, and MF128 all had slightly higher cloning efficiencies (see Figure 11) because the plates were loaded using the micropipettor to load each well individually. Experiments MF116, MF120, MF121, MF123, MF124, and MF125 all had lower cloning efficiencies (see Figure 11) because they were loaded with the sterile repeating injector and showed more mold contaminated wells than did the plates in MF63, MF63, MF82, MF115, MF126, and MF128.

The plates that I scored and obtained results from had no more than a couple of wells with mold contamination. The plates that I discarded in these experiments were completely covered in mold growth. For some reason, the sterile injector procedure was leading to mold contamination in these wells because of the difficulty I had in directing the injector into the wells. This caused the cloning efficiency to be lower in these experiments. The average cloning efficiency for all the experiments was 9% (see Table 3), which is analogous to the cloning efficiency Dr. Fondy finds using the test tube method.

*Limiting Dilution Assays in 96-Well Plates: Contents of Wells that Cloned*

In the wells that cloned, the overwhelming majority contained enlarged cells. This is not surprising, since more wells contained enlarged CB-treated cells, but it must be remembered that untreated U937 leukemia cells have a cloning efficiency of 50% to 70% and are extremely pathogenic. Of the 100 wells that cloned, 94 contained only enlarged cells and 6 contained only small cells (Note: these numbers are in no way related to the overall percentages of enlarged and small CB-treated cells that were seeded [94% enlarged to 6% small]. It is a mere coincidence that the two numbers ended up being the same and that an even 100 wells cloned). When I compare the proportion of enlarged CB-treated cells that cloned to the proportion of small CB-treated cells that cloned, the results are remarkable.

In the wells that contained multiple enlarged CB-treated cells, I determined the upper and lower limit cloning efficiencies. The upper limit

cloning efficiency is the case in which all the enlarged CB-treated cells in these wells cloned and the lower limit cloning efficiency is the case in which only one enlarged CB-treated cell in these wells cloned. All of the wells that cloned with small cells only contained one small cell, so an upper and lower limit cloning efficiency is unnecessary to determine.

*Limiting Dilution Assays in 96-Well Plates: Cloning Efficiency Range of Enlarged, Multinucleated CB-Treated U937 Cells*

The enlarged CB-treated cells still have a cloning efficiency that is far lower than that of untreated U937 cells (4% to 11% compared to 50% to 70%, respectively). This means that CB-treatment is altering the viability and clonogenicity of U937 leukemia cells that disrupt the actin filaments. If none of the enlarged cells were clonogenic after treatment with CB, then it would mean that they were dead and not potential targets for destruction. The CB treatment alone would have killed all the pathogenic cells. The fact that at least some are still viable indicates that they can be targets for further treatment leading to complete destruction.

The 4% to 11% (or more in agarose) of these enlarged CB-treated cells that remain viable and potentially pathogenic are our targets. If these cells are ‘super clonogenic,’ then they have the potential to be extremely pathogenic because they are multinucleated and may produce multiple viable daughter cells when they divide. Exposing these viable enlarged, multinucleated CB-treated

leukemia cells to sonication or X-radiation, may kill the enlarged cells that remain viable. This could then be used as a therapeutic approach in cancer treatment.

*Limiting Dilution Assays: Cloning Efficiency of Small, Mononucleated U937 Cells in CB-Treated Populations*

The small, mononucleated CB-treated leukemia cells should have a 50% to 70% cloning efficiency if they are simply U937 leukemia cells unaffected by Cytochalasin B treatment or if they are U937 cells that were able to successfully complete both mitosis and cytokinesis in spite of having disrupted actin microfilaments. The small cells present in the CB-treated population have a 13% cloning efficiency, which is significantly lower than the 50%-70% of untreated small leukemia cells, indicating that the small CB-treated cells are in some way affected by CB treatment. If they went through the cell cycle and divided without being altered, they should be clonogenic. This means that the small cells seen in the CB-treated population could be small cells because they are dead or they could be small non-clonogenic cells because they are neutrophils or other types of differentiated cells.

*Proposed Improvements for Procedures Used in These Experiments*

Some advances and improved techniques can be built off of these methods and results. More experiments need to be run and data needs to be gathered on cloning the cells in agarose in each well of a 96-well plate and centrifuging the contents down to the same microscopic plane. I did test this technique by

methods I have explained in the work presented above, but have not yet performed experiments scoring enlarged cells using this procedure. Unlike with limiting dilution in liquid medium, if more than one cell is seeded in each well in agarose then it would be possible to determine which cell was responsible for the cloning, as long as the plates were scored before the clones spread throughout the entire well.

If the limiting dilution assays in the 96-well plates in liquid medium method is the preferred method, then it would be necessary to obtain a dilution so that there is less than one cell per well. The dilution I have now, gives 1.5 cells per well, but that is not always what is seen. The dilution would have to be calculated to get less than one cell per well. More plates would have to be seeded because more wells would be empty. There is no guarantee, either, that one would seed only one cell per well. For this reason, a distribution for cloning efficiency will always be seen (see Appendix A).

Another advancement that could be made would be to determine the size of the enlarged cells to see if we can relate enlarged cell size to the degree of clonogenicity. One would be able to determine if there is a correlation between cell size and clonogenicity by determining the actual sizes of the cells in the wells. One would be able to tell if an enlarged cell was 22u or 26u, etc. by using the circular eyepiece reticle in the Reichert microscope to determine the cell sizes.

For more comparison purposes, it would be beneficial to seed equal proportions of enlarged and small cells to see how more of the small cells act. I did not work with large proportions of small cells, since that was not the focus of

my research, but it would be helpful to gather that data. I was able to successfully work with populations of enlarged, multinucleated cells. It was important to see the way enlarged, multinucleated cells act, but now that data must be expanded upon.

It would also be important to determine how many nuclei each enlarged cell has and how many daughter cells it goes on to produce. One needs to know if a multinucleated cell is initially producing two daughter cells that are initially multinucleated after one cytokinesis event or if it producing multiple daughter cells that may be mononucleated after multiple simultaneous cytokinesis events. These multinucleated cells have the potential of being super clonogenic and it needs to be determined if they are producing multiple viable daughter cells.

*Proposals for the Therapeutic Treatment of Clonogenic Enlarged Multinucleated Leukemia Cells Produced by Cytochalasin B Treatment*

In regards to using Cytochalasin B as a therapeutic agent, a next step would be to X-ray or sonicate the neoplastic cells treated with CB and then compare the cloning efficiency of these cells to the cloning efficiency of CB treated neoplastic cells not X-rayed or sonicated. X-ray and sonication are two ways in which neoplastic cells can be damaged. If our proposal is correct, the CB-treated neoplastic cells exposed to X-ray or sonication will have a lower cloning efficiency than the CB-treated cells not exposed to X-ray or sonication.

If the proposal that CB treated neoplastic cells exposed to X-ray or sonication show a lower cloning efficiency than normal neoplastic cells can be



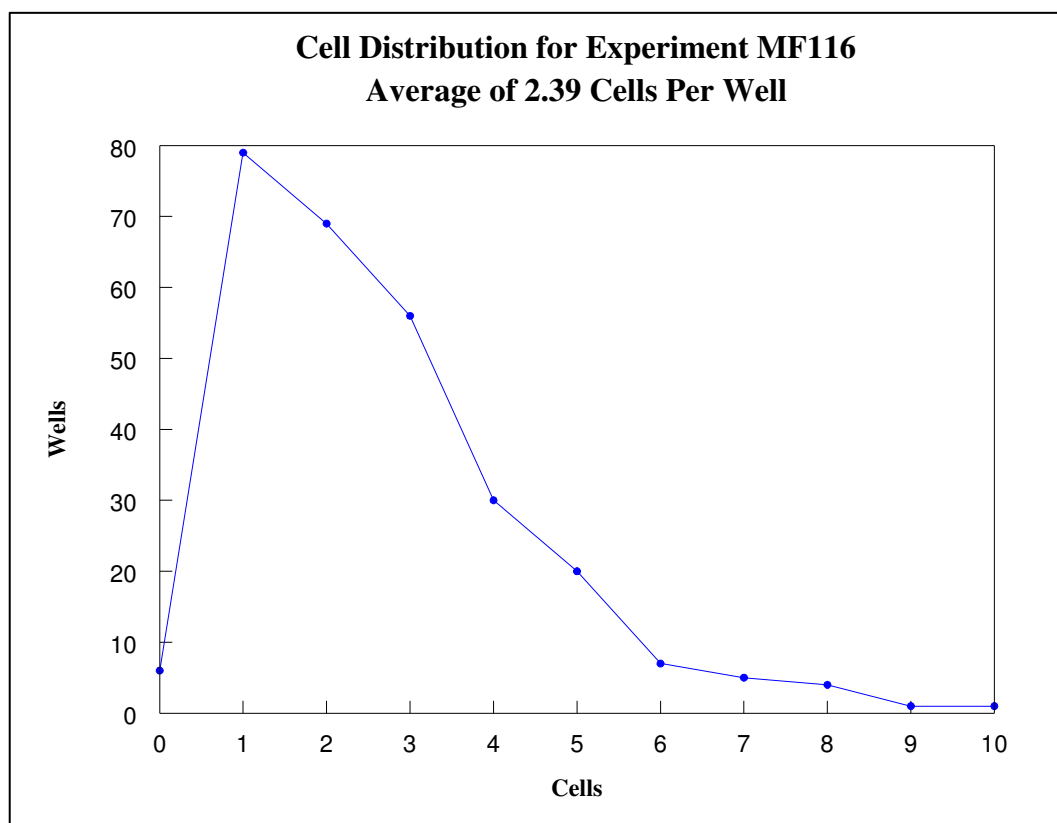
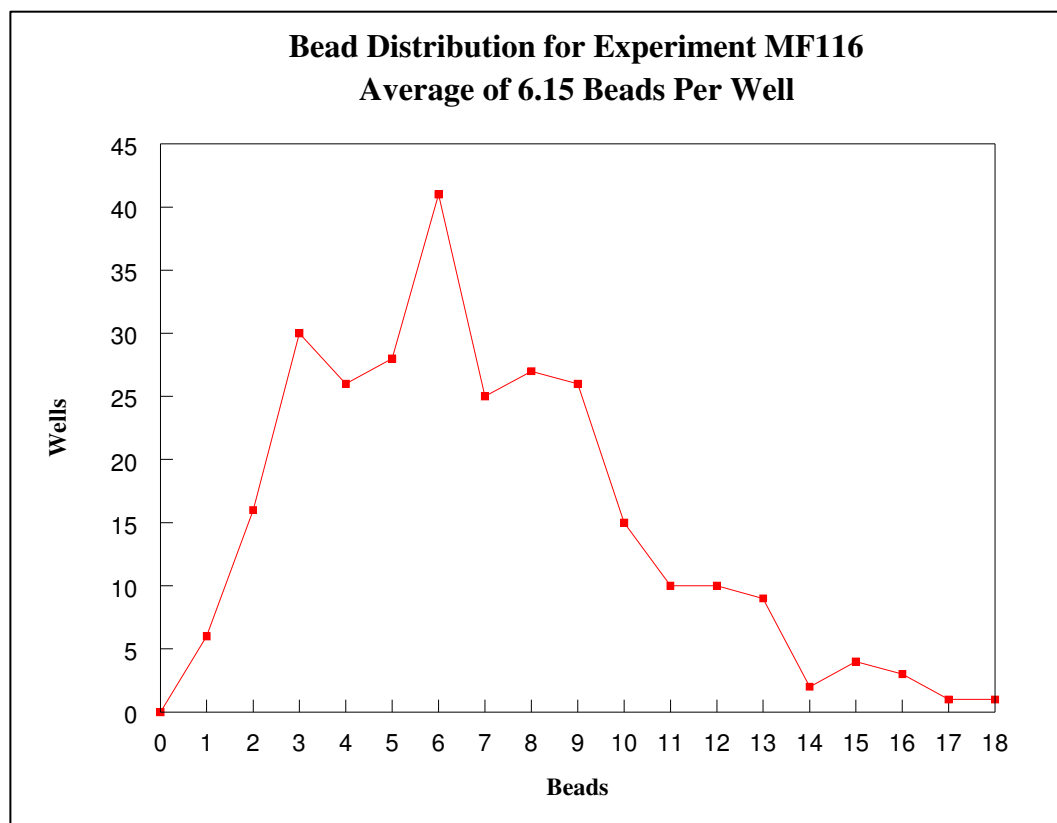
confirmed, this could potentially introduce Cytochalasin B treatment as a therapeutic agent in leukemia treatment. Cytochalasin B treatment, combined with the physical and chemical modalities of X-ray and sonication, could enhance and increase the cytotoxic effects of microfilament agents in cancer therapy. This work shows the enlarged multinucleated CB-treated U937 cells retain viability and clonogenicity when the CB is diluted out and thus remain potentially pathogenic and are targets for therapeutic modalities based on increased size and multinucleation.

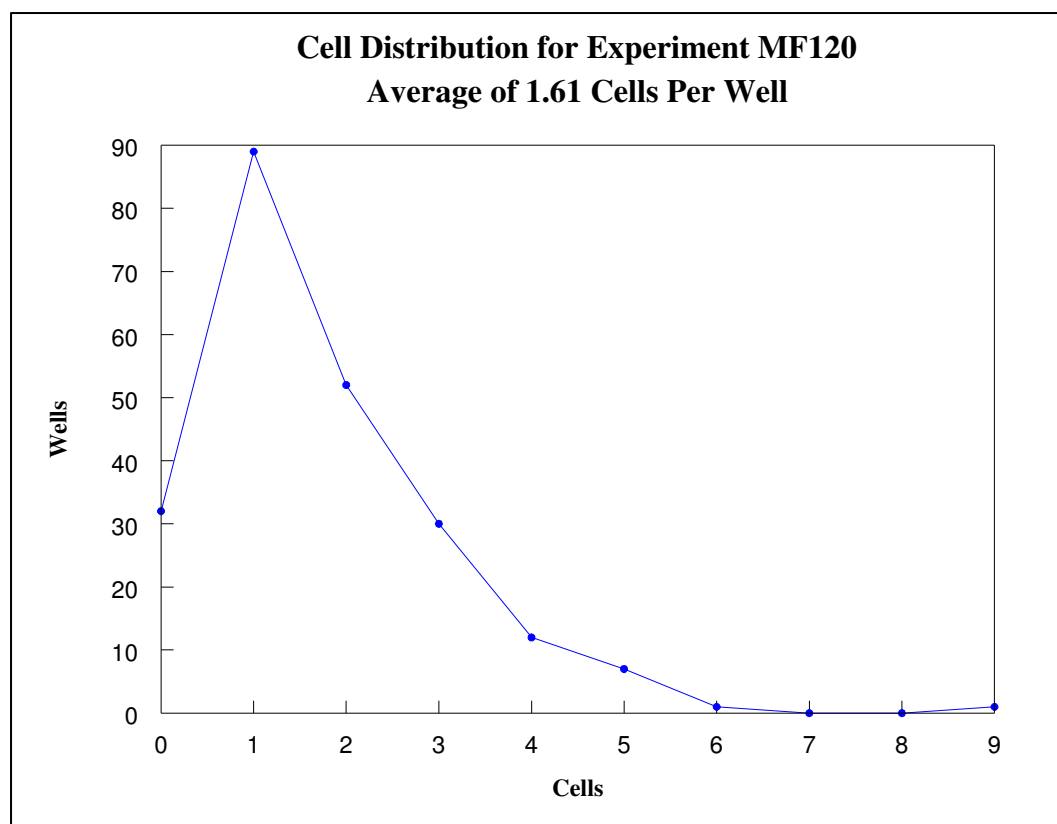
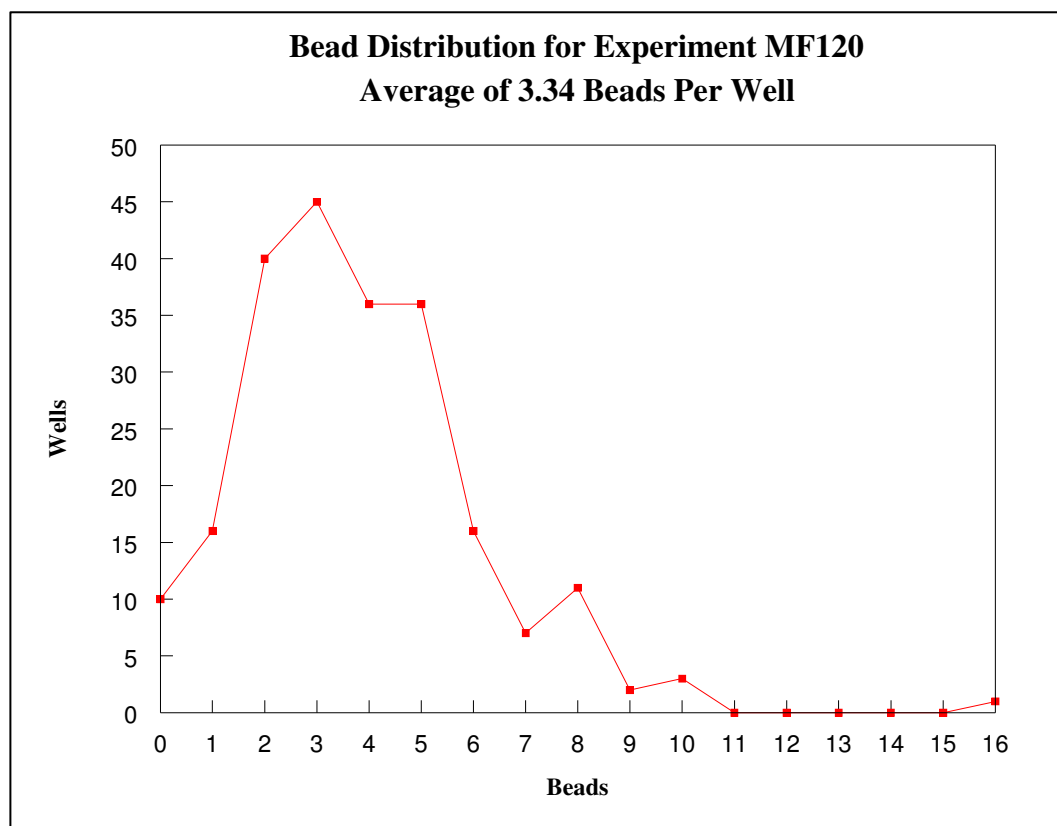
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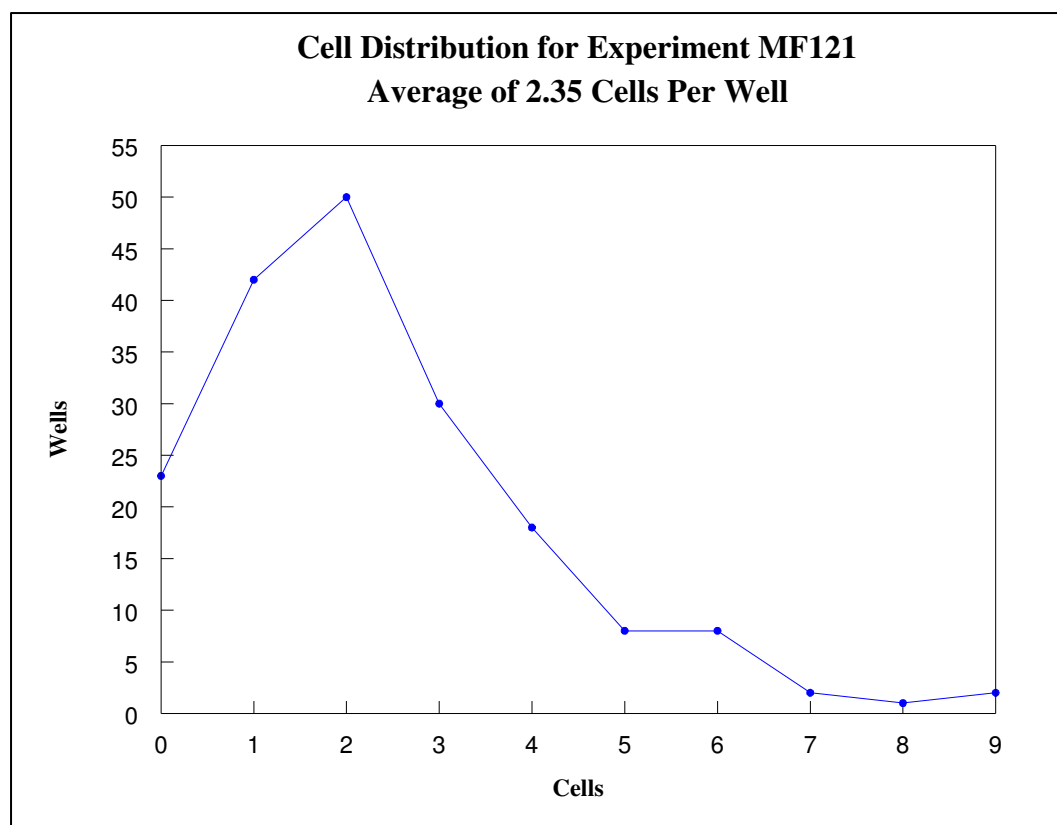
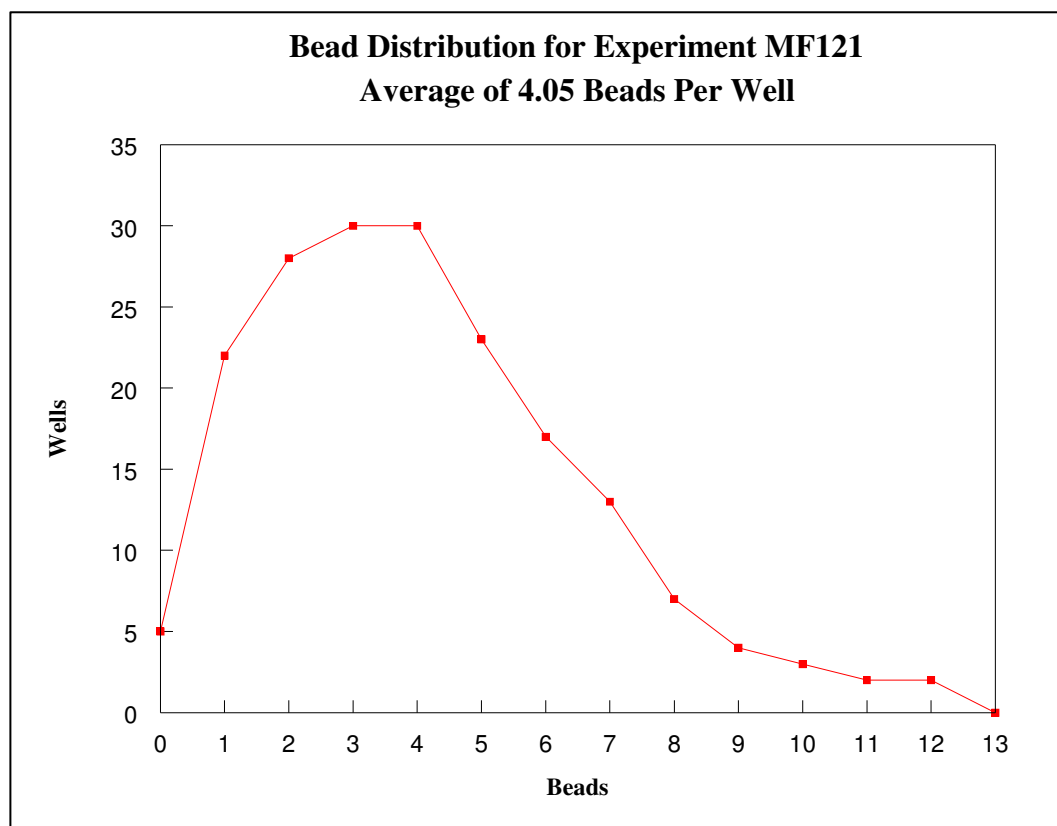
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**Appendices:** Appendix A: Cell and Bead Distributions for Some Experiments







## Appendix B: Data Used to Determine Results.

**Data used if I excluded wells that contained mixed enlarged and small cells.**

<b>Experiment</b>	<b>Total Wells</b>	<b>Wells cloned</b>	<b>Cloning Efficiency</b>	<b># of plates scored</b>	<b># of enlarged cells</b>	<b># of small cells</b>
<b>MF63</b>	16	2	12.5	1	8	8
<b>MF82</b>	99	7	7.07	2	139	6
<b>MF115</b>	50	15	30	1	66	6
<b>MF116</b>	289	6	2.08	3	633	1
<b>MF120</b>	192	10	5.21	2	242	3
<b>MF121</b>	49	1	2.04	1	102	3
<b>MF123</b>	68	4	5.88	1	78	10
<b>MF124</b>	75	4	5.33	1	129	3
<b>MF125</b>	72	1	1.39	1	185	2
<b>MF126</b>	60	16	26.67	1	172	2
<b>MF128</b>	161	34	21.12	2	373	2
<b>Sums</b>	1131	100	8.84	16	2127	46

**Data used if I included wells that contained mixed enlarged and small cells.**

<b>Experiment</b>	<b>Total Wells</b>	<b>Wells cloned</b>	<b>Cloning Efficiency</b>	<b># of plates scored</b>	<b># of enlarged cells</b>	<b># of small cells</b>
<b>MF63</b>	16	2	12.5	1	8	8
<b>MF82</b>	115	11	9.57	2	166	29
<b>MF115</b>	50	15	30	1	66	6
<b>MF116</b>	315	7	2.22	3	696	43
<b>MF120</b>	192	10	5.21	2	242	3
<b>MF121</b>	55	1	1.82	1	121	10
<b>MF123</b>	69	4	5.8	1	80	11
<b>MF124</b>	75	4	5.33	1	129	3
<b>MF125</b>	74	1	1.35	1	187	5
<b>MF126</b>	69	21	30.4	1	196	11
<b>MF128</b>	169	37	21.89	2	392	11
<b>Sums</b>	1199	113	9.42	16	2283	140